STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

REPRINTS

VOLUME XLIII

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CHANGE OF ACID AGGLUTINATION OPTIMUM AS INDEX OF BACTERIAL MUTATION.

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INTRODUCTION.

Two distinct varieties of microbe have been shown to exist in cultures of the bacillus of rabbit septicemia (1). These have been designated as Microbes D and G. Microbe D is the variety isolated from rabbits dead of spontaneous infection with the rabbit septicemia bacillus. It is characterized by diffuse growth in serum and plain broth, forms opaque fluorescing colonies on serum agar, and is highly virulent for rabbits. Microbe G, first discovered accidentally in Microbe D cultures, has been proved to be a true mutant of the parent D form (2). The mutant Microbe G grows in granular fashion in liquid media, forms translucent bluish colonies with no fluorescence, and exhibits extremely low virulence for rabbits. The mutation experiments demonstrating that Microbe D, under controllable conditions, changes into Type G were performed with D strains arising from single individuals isolated by Barber's pipette.

The granular growth of Microbe G in fluid medium is one of its most striking differential characters, and has persisted throughout transplants for more than 1 year. This sedimenting growth of Type G in broth, compared to the evenly suspended, uniformly turbid appearance of broth cultures of Type D led to an examination of the acid agglutination optima of the two types.

Methods.

The method for the determination of the acid agglutination optimum was that of Michaelis (3), later described in full by Beniasch (4). It consisted in mixing carefully prepared suspensions of the organism

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to be tested with equal volumes of buffer mixtures of varying C_{H^+}. Two buffer series were employed, Na lactate-lactic acid and Na acetate-acetic acid. The mixtures were made according to Tables I and II.

TABLE I.

Na Lactate-Lactic Acid Series.

pH	4.7	4.5	4.1	3 8	3 5	3.3	3.0	2.7	2.4
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Na Lactate N/10.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lactic acid N/10	0.06	0.12	0.25	0.5	1.0	—	—	—	—
Lactic acid N	—	—	—	—	—	0.2	0.4	0.8	1.6
Distilled water.......	1.54	1.48	1.35	1.1	0.6	1.4	1.2	0.8	—

TABLE II.

Na Acetate-Acetic Acid Series.

pH	5.6	5.35	5.05	4 75	4.4	4.1	3.8	3.5	3 2	
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	
Na Acetate N/10..........	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Acetic acid N/10	0.06	0.12	0.25	0.5	1.0	—	—	—	—	Final vol-
Acetic acid N	—	—	—	—	—	0.2	0.4	0.8	1.6	ume, 2.1 cc.
Distilled water............	1.54	1.48	1.35	1.1	0.6	1.4	1.2	0.8	—	

Preparation of Suspensions.

Microbe G sediments rapidly in fluid media. It fails to remain in even suspension when the sediment from a centrifuged culture is taken up in 0.85 per cent NaCl. If, however, such sediments are repeatedly washed in large volumes of distilled water, very stable suspensions can be obtained. In order to secure perfect comparability, Microbe D was treated in a similar manner, although in this case washing with distilled water is unnecessary and it yields stable suspensions in 0.85 per cent NaCl. The technique of preparation of suspensions was as follows.

5 per cent rabbit serum broth cultures, 24 hours incubation, were centrifuged, the supernatant fluids discarded, and the sediments thoroughly shaken in a volume of distilled water equal to that of the

original culture. The centrifugation and resuspension in distilled water were repeated four times in all. The final suspensions were carefully brought to a uniform turbidity.

EXPERIMENTAL.

All experiments were carried out by adding 1 cc. of distilled water suspension of the microbe in question to an equal volume of each of the buffer mixtures just described. The tubes were carefully shaken, placed in the water bath at 43°C. and readings taken at 1, 2, and 16 hours.

TABLE III.

Acid Agglutination Optimun of Microbes D and G, Strain R 15.
Na Lactate-Lactic Acid Buffer Series.

Organism.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH. Final reading.
	pH	4.7	4.5	4 1	3.8	3.5	3.3	3.0	2 7	2.4	
Microbe D.	1 hour.	0	0	0	0	+	+	0	0	0	3.5–3.3
	2 hours.	0	0	0	0	++	++	+	+	Tr.	
	16 "	0	0	0	0	C	C	+	Tr.	Tr.	
Microbe G.	1 hour.	Tr.+	+	+	++	+	0	0	0	0	4.7–3.8
	2 hours.	Tr.+	++	++	C	++	0	0	0	0	
	16 "	C	C	C	C	+	Tr.+	0	0	0	.

In this and the following tables lesser degrees of flocculation are recorded as ++, +, and Tr. (trace). C indicates complete flocculation.

The agglutination optimum was considered to be that zone of C_H^+ where complete flocculation occurred; that is, where the microbes sedimented so perfectly as to leave a water-clear supernatant fluid. Lesser degrees of agglutination are recorded as ++, +, and Tr. (trace). The readings are greatly facilitated by holding the tubes before a powerful beam of light, projected downward in front of a dark background.

Experiment 1. Acid Agglutination Optimum, Microbes D and G, Strain R 15, in Na Lactate-Lactic Acid Buffer Series.—Suspensions of Microbes D and G, isolated from Strain R 15, bacillus of rabbit septicemia, were tested against the Na lactate-lactic acid buffer series. The experiments were carried out as described above. The results are recorded in Table III.

The results recorded in Table III show a definite difference in acid agglutination optimum of the two varieties. The readings, taken at 1,2, and 16 hours, indicate that the reaction does not take place with the speed of that of immune agglutination of the majority of bacteria. It is necessary to allow ample time to elapse before taking the final readings. 16 hours have been found to be sufficient, no material change in readings being noted after this time.

The same suspensions were tested against the Na acetate-acetic acid buffer series, with a similar result recorded in Table IV. Temperature, as before, was 43°C. in the water bath.

TABLE IV.

Acid Agglutination Optimum of Microbes D and G.
Na Acetate-Acetic Acid Series.

Organism.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH.
	pH	5.5	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
Microbe D.	1 hour.	0	0	0	0	0	0	0	+	+	3.5 –3.2
	2 hours.	0	0	0	0	0	0	0	C	C	
	16 "	0	0	0	0	0	0	0	C	C	
Microbe G.	1 hour.	0	0	0	+	++	++	+	0	0	4.75-3.8
	2 hours.	0	0	0	++	C	C	++	0	0	
	16 "	Tr.+	Tr.	++	C	C	C	C	+	Tr.	

The results in the case of the Na acetate-acetic acid series correspond to those of the Na lactate-lactic acid mixtures. The final readings are identical, the only difference lying in a slightly more rapid flocculation in the acetate series.

Variability of Optimum of Microbe G as Compared to that of Microbe D.

A number of strains were now collected, the D and G types isolated and subjected to test with the Na acetate-acetic acid series. The result (Table V) confirms the findings of the previous experiments. Final reading was made after 16 hours with incubation at 43°C. The results are presented in Table V.

It will be noted that the acid agglutination optimum for Microbe D is the same in case of all three of the strains tested. On the other hand, the optimum for Microbe G varies to a considerable extent. This variation is never so great as to prejudice its value as a criterion of differentiation from the parent D form. In all cases complete flocculation of Type G occurs at a distinctly lower C_H^+ than that of Type D. The difference between the two types in regard to the smallest amount of hydrogen ion in which complete flocculation takes place is never less than 0.6 pH. In short, the organism in the process of mutation gains in sensitivity to flocculation in the presence of H ions.

TABLE V.

Acid Agglutination Optima of D and their Mutant G Forms.
Na Acetate-Acetic Acid Series.

Strain.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH.
	pH	5.6	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
R 15	D-S 49	0	0	0	0	0	0	0	C	C	3.5 –3.2
	G-S 52	Tr.	Tr.	++	C	C	C	C	+	Tr.	4.75–3.8
	G-S 28*	Tr.	Tr.	C	C	C	C	C	++	++	5.05–3.8
R 11	D-S 43	0	0	0	0	0	0	0	C	C	3.5 –3.2
	G-S 42	0	0	+	+	+	C	C	C	+	4.1 –3.5
R 22	D-S 31	0	0	0	0	0	0	Tr.	C	C	3.5 –3.2
	G-S 32	0	Tr.	+	+	C	C	C	++	Tr.	4.4 –3.8

* G-S 28, a mutant from the same parent D strain as G-S 52.

Sobernheim and Seligmann (5) found a strain of *Bacillus enteritidis* to separate into two races. Beniasch (4) tested the acid agglutination point of this organism and found it to have altered its acid agglutination optimum when tested on two different occasions, a year having elapsed between the two tests. In this work apparently no attempt was made to establish the occurrence of a mutation, or to separate the two varieties.

Variations in the Agglutination Optimum of Type G.

Table V indicates that the agglutination optima of various strains of Microbe G are not as strictly uniform as those of the parent D type. One of the causes of this variation is passage of the microbe through the animal body. An example of this variation was observed during an attempt to cause reversion of Microþe G to the parent D form.

The Type G strain in question was characteristically of very low virulence. 1.0 cc. of a serum broth culture, injected intrapleurally, was required to produce fatal infection of a 600 gm. rabbit. The organism recovered at necropsy of this animal was cultured and injected into a second animal, and so on. At the third animal passage

TABLE VI.

Effect of Animal Passage on Acid Agglutinability of Type G.

	pH'	5 6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2
Microbe R 15 G	Before passage.	Tr.	Tr.	++	C	C	C	C	+	Tr.
	After three animal passages.	C	C	C	C	C	C	C	C	C

the virulence had greatly increased, 10^{-4} cc. of a serum broth culture being fatal. But the organism, far from returning to the uniformly turbid growth character of the Type D form, became more intensely granular 'in its growth. This characteristic was so marked that difficulty was experienced in preparing the washed suspensions for acid agglutination test.

The acid agglutination reaction of Type G strain after animal passage was compared with the same strain which had been transplanted in parallel in serum broth. The Na acetate-acetic acid buffer series was used. The culture was incubated at 43°C. for 16 hours (Table VI).

It will be seen from Table VI, first, that much less hydrogen ion is required to produce complete flocculation, and second that the optimum is very greatly broadened. It has been widened from

pH 4.7 to 3.8 before animal passage, to pH 5.6 to 3.2 after passage through three rabbits.

Up to the present, the change in acid agglutination optimum that occurs during mutation has been accompanied invariably by a great loss in virulence. For example, all Type D strains tested have been fatal to rabbits in doses of 10^{-5} to 10^{-7} of a serum broth culture. The Type G forms arising from such strains are seldom fatal in 0.5 cc. of undiluted culture. Frequently rabbits are able to resist 1.0 cc.

The experiment just described indicates that the decrease in stability to acid does not necessarily go hand in hand with loss of virulence, and certainly bears no causal relationship to such loss. For, while the stability to the hydrogen ion had greatly *decreased* during animal passage, the virulence had *increased* from 0.5 cc. to 10^{-4} cc.

SUMMARY AND CONCLUSIONS.

A distinct difference in acid agglutination optimum for Type D (bacillus of rabbit septicemia) and its mutant form, Type G, has been observed. The optimum for Type D lies between pH 3.5 and pH 3.0. This changes during mutation, the resulting Type G mutants having in general an optimum lying between pH 4.7 and pH 3.8.

The constancy of the optimum for Type D is very strict, while that for Type G is slightly less so. The variation is never so great as to cause an overlapping of optima and consequent failure of differentiation.

These acid agglutination optima are in the nature of physical constants for the two types and would imply a fundamental difference in the chemical constitution of the organisms.

Animal passage, far from causing a reversion of the mutant Type G to the primordial Type D form, brings about a still greater instability in the presence of H ions.

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THE MECHANISM OF GRANULAR GROWTH OF RABBIT SEPTICEMIA BACILLUS TYPE G.

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INTRODUCTION.

It has been stated by Beniasch (1) that the acid flocculation optimum of bacteria is referable only to the C_H+ and is not influenced by the unionized acid or the anion of the acid. This concept may hold for buffer mixtures made up of certain acids and their sodium salts. But certain buffer mixtures exist which are composed of an acid plus some totally different substance, bearing no close chemical relationship to the acid. Examples of such buffer mixtures are glycocoll-HCl and glycocoll-Na acetate-NaH_2PO_4-HCl. These have been found by the writer (2) to be very useful in acid agglutination studies. It is important to learn whether such buffer substances exert an effect on the acid agglutination point of bacteria, which might differ from the values found for the Michaelis (3) series.

EXPERIMENTAL.

The Na lactate-lactic acid buffer series of Michaelis (3) covers a range from pH 4.7 to 2.4. It was desired to test the behavior of distilled water suspensions of Microbe G and D in higher C_H+. Since this range was not covered by the Na lactate-lactic acid series, recourse was had to the glycocoll-HCl series of Sörensen (4), which covers a range from pH 3.0 to pH 1.2. This buffer series was prepared from the Sörensen chart.

The pH of these mixtures was tested colorimetrically and checked by the potentiometer.

The flocculating activity of this buffer series was then compared to that of the Na lactate-lactic acid series. The technique of the

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experiments was identical with that described in the preceding paper (5). 1 cc. of the buffer mixtures was added to equal volumes of four times washed distilled water suspensions of Microbes D and G, bacillus of rabbit septicemia. The mixtures were placed in the water bath at 43°C. for 16 hours, and readings taken. The results of this experiment are recorded in Table I.

TABLE I.

Agglutination of Microbes D and G in Na Lactate-Lactic Acid and Glycocoll-HCl Buffer Series.

	pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4		Complete agglutination.
Na lactate-lactic acid.	Microbe D.	0	0	0	++	C	C	+	+	0		*pH* 3.5–3.3 None at 2.7–2.4.
	Microbe G.	C	C	C	++	+	0	0	0	0		4.7–4.1 None at 3.3–2.4.
	pH	3.0	2.8	2.6	2.4	2.2	2.0	1.8	1.6	1.4	1.2	
Glycocoll-HCl.	Microbe D.	C	C	C	C	++	++	++	++	+	0	3.0–2.8
	Microbe G.	++	++	+	+	+	0	0	0	0	0	++ 3.0–2.8

In this and the following tables the mixtures were kept in the water bath for 16 hours at 43°C. C indicates complete flocculation; Tr., trace.

The results given in Table I indicate that other factors besides the C + are important in the interpretation of the acid agglutination point of the organism in question. In the case of Microbe D, Na lactate-lactic acid series, for example, complete flocculation occurs at pH 3.5 to 3.3, slight at pH 3.0, a trace at pH 2.7, and none at all at pH 2.4..

On the other hand, Microbe D in the glycocoll-HCl series, flocculates completely at pH 3.0, 2.8, 2.6, and 2.4. This difference of effect in the two buffer series is illustrated graphically in Fig. 1.

A similar result occurs in the case of Microbe G which flocculates completely at pH 4.7, 4.5, and 4.1, and not at all at pH 3.0 to 2.4, in the Na lactate-lactic acid series. Yet ++ agglutination occurs at

pH 3.0 and 2.8 and + at pH 2.6 to 2.2 of the glycocoll-HCl series. It would appear that the glycocoll or the anion of the acid has the property of broadening the acid agglutination optima of Microbes D and G, or of shifting the optima toward a zone of higher $C_H{}^+$.

These results led to experiments which suggest an explanation for the granular growth of Microbe G in plain broth. Washed suspensions of this organism in distilled water, pH 6.0, do not sediment. On the other hand, rapid sedimentation occurs in plain broth at pH 7.4 to 7.0. This leads to the conclusion that broth contains a constituent which, *per se*, agglutinates the Type G organisms, or which has the property, like glycocoll, of shifting their acid agglutination optimum.
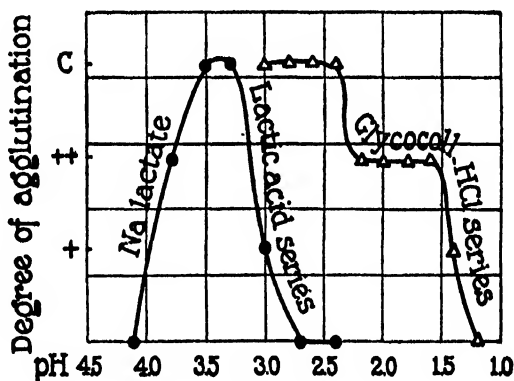
FIG. 1.

Preliminary experiments were made in which the flocculating effect of the various components of plain broth were tested against washed distilled water suspensions of Microbe G. The constituents tested were beef infusion, peptone, and Na_2HPO_4.

Beef Infusion.—500 gm. of chopped beef were extracted in 1,000 cc. of tap water, in the ice box for 16 hours. The mixture was then boiled for 30 minutes, filtered, titrated to pH 7.4, refiltered, and sterilized in the autoclave.

Peptone Solution.—10 gm. of Fairchild's peptone were dissolved in 1,000 cc. of distilled water, boiled for 30 minutes, filtered, adjusted to pH 7.4, and sterilized in the autoclave.

Na_2HPO_4.—10 gm. of this salt were dissolved in one liter of distilled water adjusted to pH 7.4, and sterilized in the autoclave.

Of these three solutions, at pH 7.4, beef infusion alone showed marked flocculating activity against Microbe G.

The flocculating effect of beef infusion at varying acidities was now tested. The beef infusion solution just described was titrated with N/10 HCl to increasing degrees of acidity, from pH 7.5 to pH 2.0. The beef infusion at all of these C_H+ was now diluted with distilled H_2O, the dilutions increasing from 1:2 to 1:40. Each of these dilutions at each pH, was now added in equal volume to 1 cc. of distilled water suspensions of four times washed Microbes D and G. The mixtures were kept in the water bath for 16 hours at 43°C., and readings taken. The results are recorded in Tables II and III.

TABLE II.

Beef Infusion Agglutination of Microbe G at Varying C_H+.

pH	Amount of beef infusion.							Result.
	1.0 per cent	0.75 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	
7.5	++	++	++	+	+	0	0	
7.0	C	++	++	++	+	+	0	
6.5	−	C	C	++	++	+	+	
6.0	−	C	C	++	++	++	+	Final volume of beef infu-
5.0	−	C	C	C	C	++	+	sion dilution = 1.0 cc.
4.5	−	C	C	C	C	C	C	+ distilled H_2O suspen-
4.0	−	C	C	C	C	C	C	sion G 1.0 cc.
3.5	−	C	C	C	++	++	+	
3.0	−	C	C	C	++	++	+	
2.5	−	C	C	++	++	+	+	
2.0	−	C	C	++	+	+	+	

Table II shows for Type G, that, as the acidity increases down to to pH 4.5, the amount of beef infusion necessary to cause complete agglutination becomes less and less. At pH 4.5 to pH 4.0 traces of beef infusion cause complete agglutination. This point corresponds to the acid agglutination optimum of Microbe G in various buffer series. Beyond this point, that is, at pH < 4.0, increasing amounts of beef infusion are again necessary to produce complete flocculation.

Table III shows the same effect in the case of Microbe D, the only difference being that complete flocculation of the D type by a given

concentration of beef infusion demands a higher C_H+ than in the case of Type G. For each organism the range of C_H+ at which the smallest amount of beef infusion is required for complete sedimentation is precisely the optimum zone of acid agglutination described in the preceding paper.

The results of the experiments recorded in Tables II and III are represented graphically in Fig, 2, in which the pH of the various dilutions of beef infusion are plotted on the abscissæ against the *recip-*

TABLE III.

Beef Infusion Agglutination of Microbe D at Varying C_H+.

pH	Amount of beef infusion.							Result
	1.0 per cent	0.75 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0 075 per cent	
7.5	Tr.	0	0	0	0	0	0	
7.0	+	+	+	Tr.	0	0	0	
6.5	—	C	C	++	+	0	0	Final volume of beef infu-
6.0	—	C	C	++	+	Tr.	Tr.	sion dilution = 1.0 cc. +
5.0	—	C	C	C	++	+	+	distilled H_2O suspension
4.5	—	C	C	C	C	++	++	D 1.0 cc.
4.0	—	C	C	C	C	C	C	
3.5	—	C	C	C	C	C	C	
3.0	—	C	C	C	++	++	++	
2.5	—	C	C	C	++	++	++	
2.0	—	C	C	++	++	+	+	

rocals of the amount of beef infusion on the ordinates. That is, 1.0 cc. of beef infusion is represented by 1, 0.5 cc. by 2, 0.5 cc. by 4, and so on. The points recorded on the graphs are in all cases the amounts of beef infusion which cause *complete flocculation*, represented by C, Tables II and III.

The graphs of Fig. 2 would seem to afford an explanation for the granular growth of Microbe G and the diffuse growth of Microbe D in broth, since at pH 7.0, the C_H+ at which these organisms are grown, large amounts of beef infusion cause complete sedimentation of Microbe G and little or no agglutination of Microbe D.

What is more, the graphs would appear to indicate that beef infusion, *per se*, does not cause the agglutination. It merely widens the
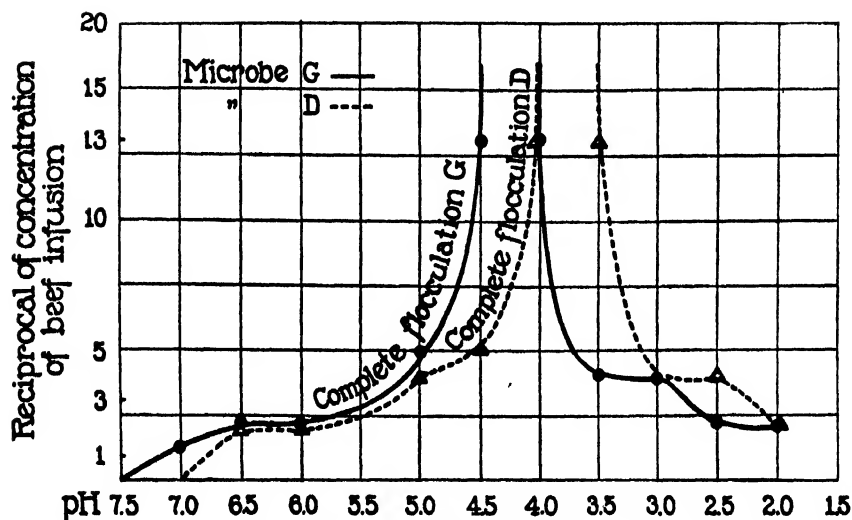
FIG. 2.

acid agglutination optimum zone. This is clear from the fact that small amounts of beef infusion do not cause a flocculation independent of the C_H+.

Suspensions of Types D and G were similarly tested against decreasing concentrations of peptone (Fairchild) at various C_H+. The technique of the experiments was identical with that for beef infusion. The peptone solution from which the various dilutions were made

TABLE IV.

Peptone Agglutination of Microbe G at Varying C_H+.

pH	Amount of peptone.							Result.
	1.0 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	0.05 per cent	
7.5–6.0	0	0	0	0	0	0	0	
5.0	C	++	Tr.	0	0	0	0	
4.5	C	C	Tr.	0	0	0	0	Final volume of peptone
4.0	C	C	C	C	++	Tr.	0	·dilutions 1.0 cc. + dis-
3.5	C	C	C	C	C	C	+	tilled H_2O suspension
3.0	C	C	C	C	C	C	C	G 1.0 cc.
2.5	C	C	C	C	C	C	C	
2.0	C	+	+	Tr.	0	0	0	

was of 1 per cent concentration in distilled water. The results are recorded in Tables IV and V.

TABLE V.

Peptone Agglutination of Microbe D at Varying C_H+.

pH	Amount of peptone.							Result.
	1.0 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	0.05 per cent	
7.5–3.5	0	0	0	0	0	0	0	Final volume of peptone
3.0	0	0	C	C	C	C	Tr.	dilutions 1.0 cc. + dis-
2.5	0	C	C	C	C	C	C	tilled H₂O suspension
2.0	0	0	C	C	++	++	++	D 1.0 cc.

FIG. 3.

It will be observed from Tables IV and V that the results are strikingly different from those obtained with beef infusion. Peptone contrary to beef infusion, appears to shift the optimum zone of agglu tination in the direction of a higher C_H+, and effect analogous to tha observed in the glycocoll-HCl buffer mixtures (Table I).

The optimum for Type G in the lactate and acetate buffer mixture lies between pH 5.0 and pH 3.8. Peptone changes the optimum to pH 3.0 to 2.5. For Type D, an analogous effect is observed, th

optimum shifting from pH 3.5 to 2.5. The results recorded in Tables IV and V are graphically represented in Fig. 3.

It is interesting to observe that for Microbe D strong concentrations of peptone (0.5 and 0.25 cc. of 1 per cent solution) actually suppress flocculation completely at pH 3.0. The effect of peptone, contrary to that of beef infusion, would appear to be a stabilizing one.

SUMMARY.

The acid agglutination optimum of Microbes D and G is not independent of the nature of the buffer mixture. Glycocoll-HCl buffer mixtures cause complete flocculation at high C_H+ (2.7 to 2.4), at which points little or no flocculation occurs with the Na lactate-lactic acid buffer series.

Beef infusion has the property of broadening the acid agglutination optimum of both Microbes D and G, bacilli of rabbit septicemia. This extension is in the direction of a lower C_H+.

There is no evidence that the beef infusion has the power, *per se*, of agglutinating these organisms. It would seem merely to increase their sensitiveness to sedimentation in the presence of H ions.

The data presented explain the mechanism of the granular growth character of Microbe G in liquid media as compared to the diffuse growth of Microbe D.

Peptone (Fairchild), contrary to beef infusion, shifts the acid agglutination optimum of Microbes D and G in the direction of a higher C_H+. Strong concentrations of peptone exhibit an inhibitory effect on the agglutination of Microbe D in the optimum zone.

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3. Michaelis, L., *Deutsch. med. Woch.*, 1911, xxxvii, 969.
4. Sörensen, S. P. L., *Compt. rend. Trav. Lab. Carlsberg*, 1909, viii, 1; *Biochem. Z.*, 1909, xxi, 131, 201.
5. De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 387.

EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

IV. Immunizing and Toxic Agents Found Occasionally in Filtrates of Typhus-Infected Tissues.

By PETER K. OLITSKY, M.D.

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Plates 30 and 31.

In a previous article[1] we stated that typhus virus present in tissues of guinea pigs reacting to the experimental disease failed to pass through Berkefeld V and N candles. Reference was also made to the non-filterability of the virus in the blood of infected animals.

The object of this paper is to show that these filtrates, which are free, so far as we can ascertain, from a living, multiplying agent, can occasionally induce in guinea pigs not only the typical lesions of the disease but also immunity to later injections of the active virus.

Method.

The brain and spleen obtained from guinea pigs during the height of experimental infection were chosen as sources of typhus virus, because these organs contain the virus in greater concentration than the blood.[2,3]

The tissues were disintegrated, thus liberating the virus, and were suspended in Ringer's solution. The method has been described in the previous paper.[1] 1 cc. of the unfiltered suspension was injected intraperitoneally in guinea pigs, which served as controls in demonstrating the activity of the infected tissues. Other portions of the suspensions were passed through tested Berkefeld filters (V and N) and 5 to 10 cc. were similarly injected into thirty-nine test animals. The subsequent disposition of these animals was as follows:

(A) Nine were bled between the 4th and 10th days after inoculation by cardiac puncture and 3 cc. of the defibrinated blood were injected intraperitoneally

[1] Olitsky, P. K., *J. Exp. Med.*, 1922, xxxv, 121.

[2] Landsteiner, K., and Hausmann, W., *Med. Klin.*, 1918, xiv, 515.

[3] Weil, E., Breinl, F., and Gruschka, T., *Wien. klin. Woch.*, 1921, xxxiv, 459.

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into each of eighteen normal animals. This was for the purpose of disclosing any living microorganism in the filtrates.

(B) Twenty were observed for periods up to 16 days for clinical signs, especially early or late febrile reactions.

(C) Nine of Group B were reinjected with active blood from 10 to 16 days after the first inoculation. This was done to determine the immunizing power of filtered virus.

(D) Finally, ten were killed from 4 to 7 days after inoculation in order to note any changes produced in the tissues.

RESULTS.

The following is a summary of the results of the experiments.

Activity of the Virus Employed.—All the guinea pigs which were injected with the suspensions of unfiltered typhus-infected tissue showed, after an average incubation period of 7 to 8 days, a febrile reaction lasting, as a rule, for 8 days. After recovery, the animals remained resistant to later injections of active blood. Those killed at the height of reaction exhibited the characteristic histopathological changes of experimental typhus fever. With the blood obtained in this stage, a disease of similar type was produced in normal animals. No secondary or concurrent infections were found. The disease was, therefore, typical of experimental typhus fever in guinea pigs, as described in detail in another communication.[4]

Absence of Living Organisms.—The blood of guinea pigs which were inoculated with filtrates failed in each instance to induce in normal animals the typical fever observed in the controls. It must be concluded that the filtrates, in view of the failure to reproduce the disease by transmission, were free from a living, multiplying agent.[5]

Temperature Reactions.—Of the twenty guinea pigs inoculated with filtrates, fifteen revealed no disturbance of temperature. Of the remaining five, all showed a rise in temperature, above 40°C. (104°F.), for 1 to 3 days, first noted from 2 to 5 days after the inoculation.

Immunity.—Of the nine guinea pigs inoculated with filtrates and reinjected after 10 to 16 days with active typhus virus, six reacted typically to the second injection, and two showed a mild fever, lasting

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[4] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

[5] Experiments demonstrating the non-filterability of the typhus virus have been described in the previous paper.[3]

for 4 days, instead of 8 days as in the controls, which on transmission proved to be the familiar experimental typhus, while one was not affected.   Thus, the filtrates induced no immunity in six, a partial immunity in two, and a complete immunity in one of the animals.

Production of Lesions.—In four of ten guinea pigs inoculated with filtrates and killed 7 days after injection, the following lesions were found.

Macroscopic Examination.—The organs were normal in appearance, with the exception of the spleen, which was slightly enlarged and darker than is usual.   In two guinea pigs a few small discolored areas, resembling the spots of a petechial rash, were seen after stripping the corium of the skin.

Microscopic Examination.—The central nervous system showed no meningeal involvement, except in its blood vessels.   Focal accumulations of macrophages, or mononuclear cells, were noted about the capillaries and arterioles, which at some points were sufficient to form a distinct nodular mass giving rise to a peri-arteriolitis nodosa (Fig. 1).   The endothelium of the vessels, often swollen, was necrotic in some portions and proliferated in others.   Occasionally an arteriole or capillary was noted to contain occluding thrombi.   The gray matter of the brain, especially that of the midbrain, contained a few small localized hemorrhages.   The vessels here revealed lesions similar to those found in the vessels of the meninges.   Nodules were seen, always in proximity to the vessels, which consisted mainly of macrophages, or mononuclear cells, and a few polymorphonuclear cells (Fig. 2).

The heart tissue revealed no visible changes except for a few localized hemorrhages and the vascular lesions described above (Figs. 3 and 4).·

The changes in the spleen were such as to obscure the vascular lesions.   There were noteworthy congestion and leucocytic infiltration.   The lymphoid follicles were enlarged and at the center were a number of macrophages replacing to some extent the lymphoid cells.

The skin in two of the guinea pigs exhibited in the deeper layers of the corium small localized hemorrhages and the vascular lesions and nodules described above (Fig. 5).

The lesions found in these animals are characteristic of experimental typhus fever in the guinea pig.[4]

To summarize the results, it appears that in a small number of instances other effects than typhus are produced by the inoculation of filtrates from typhus-infected tissues.   In five of twenty guinea pigs, a rise in temperature occurred which lasted from 1 to 3 days. · One of nine guinea pigs remained immune and two others of this

series responded with a mild reaction, after a test injection with active virus. Finally, four of ten animals showed the characteristic lesions of the experimental disease.

DISCUSSION.

We have already demonstrated[1] that the typhus virus present in the tissues of guinea pigs at the height of their reaction to inoculation is not filterable through tested Berkefeld filters. Hence effects produced by means of filtrates of infected tissues cannot be attributed to a living, multiplying agent. The possibility of the presence of subinfective quantities of the virus in filtrates producing the results can be eliminated by the fact that whereas only infinitesimal amounts of infected brain tissue (0.005 gm.)[2] are necessary to induce the experimental infection, we have employed almost one-half of the amount of entire filtrate obtained from suspensions of all the brain tissue from 300 to 350 gm. guinea pigs. Furthermore, in our experience[1] transmission of the virus from animal to animal by means of filtrates has failed.

The experiments reported in this paper demonstrate that such filtrates can produce occasionally an early and short febrile reaction, immunity to later injections of active virus, and lesions indistinguishable from those of the typical experimental disease. From this we infer that a specific substance may be present in the tissues of infected guinea pigs and be occasionally obtainable in filtrates.

Nicollé and his associates[6] have maintained that the typhus virus is filterable for the reason that infrequently monkeys which have been inoculated with filtrates of blood, or of lice harboring the virus, have shown no definite temperature reaction, but remained resistant to a later injection of active virus. The experiments above described demonstrate that such occasional immunity following inoculations of filtrates depends on some other factor than a living, multiplying agent.

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[6] Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1910, cli, 685; 1911, cliii, 578. Nicolle, C., Blanc, G., and Conseil, E , *Arch. Inst. Pasteur Tunis*, 1914, ix, 84

CONCLUSION.

In the filtrates of typhus-infected tissues of guinea pigs can be occasionally found a substance which produces in these animals thermic reactions, lesions characteristic of experimental typhus, and, still less frequently, immunity to later injections of active virus. The general indications are that this substance is not a living organism.

EXPLANATION OF PLATES

PLATE 30.

FIG. 1. Section of the brain of a guinea pig inoculated with a filtrate of typhus-infected tissue. The vessels of the meninges show the perivascular accumulation of macrophages of the nature of a nodular formation and occlusion by thrombus of other vessels A small nodule can be seen in the gray matter near the surface. × 240.

FIG. 2. Another section of the same brain. A distinct nodule in the gray matter can be observed. × 240.

FIG. 3. Section of heart tissue from the same guinea pig, showing vascular lesions similar to those in the brain. × 240.

PLATE 31

FIG. 4. Another section of heart tissue. A nodule under higher magnification to show its structure × 560.

FIG. 5. Section of the deeper layers of the skin from the same guinea pig. At the edge of the section, toward the epithelial surface, is a small nodule indicated by an arrow; in the deeper muscular layer is a pronounced vascular lesion similar to that noted in Fig. 1. × 240.
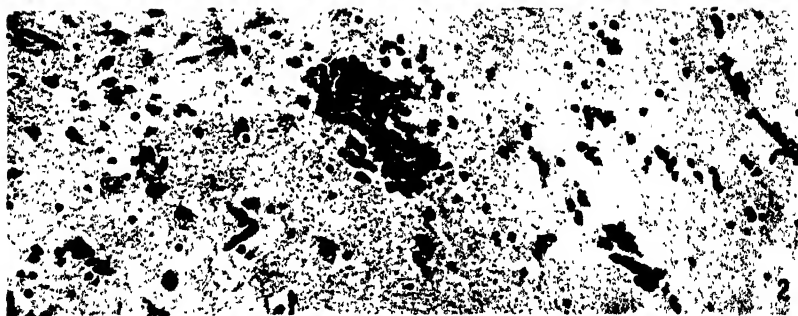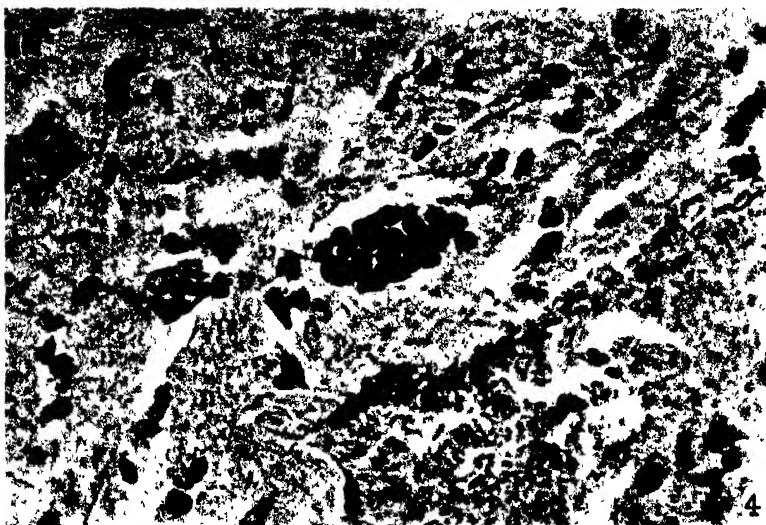
THE EFFECT OF FLOOD DIURESIS ON HEMO-GLOBINURIA.

By HERBERT HAESSLER, M.D.

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The fact is well recognized that a considerable quantity of hemo-globin must be free in the plasma if any is to pass the renal barrier and appear in the urine. The pigment is, like dextrose, a "threshold substance." It readily penetrates into the renal tubules but is absorbed again more or less completely during its course through them.[1] This being true, diuresis should diminish the chances of absorption by hastening the flow of fluid, and tend to lead to the appearance of the pigment in the urine. Evidence will here be presented that such is the case. Hemoglobinuria, like glycosuria, is much favored by flood diuresis.

Method.

A concentrated solution of hemoglobin was abruptly thrown into the circulation of rabbits and dogs, followed in some instances by a slower injection of salt solution. The amount of pigment introduced was slightly less than that required to produce hemoglobinuria in the absence of diuresis. The urine was collected at intervals by catheter.

All of the animals were males. Individuals were selected with normal kidneys, as indicated by the general character of the urine and proven by the autopsy findings.

Great care was necessary to prevent hemorrhage during the catheterization of the rabbits, and despite it a few red cells were frequently encountered after-wards in the urine. For this reason the experiments were repeated on dogs, in which the complication can be avoided. The animals were stretched out, the bladders emptied by catheter as completely as possible, and the urine set aside for

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[1] Adami, J. G., *J. Physiol.*, 1885, vi, 382.

TABLE I.

Influence of Flood Diuresis on Hemoglobinuria.

Experiment No.	Rabbit	Weight.	Hemo-globin of blood.	Per cent of total hemo-globin injected.	Amount injected.	Fol-lowed by salt solution.	Urine of next ½ hr.				Remarks.
							Amount.	Color.	Guaiac test.	Spectro-scopic bands.	
		gm.	*per cent*		*cc.*	*cc.*	*cc.*				
Rabbits.											In Experiment 1, the bloods of the animals were assumed to have the same percentage of hemoglobin, and in consequence the amounts of pigment injected differed directly as the body weight.
1	Control.	3,050	61.5	0.8	1.68	—	3.0	Yellow.	0	Absent.	
	Diuresis.	2,575	66.5	0.8	1.42	40	26.0	Pink.	+++	Present.	
2	Control.	3,050	71.0	0.8	1.9	—	4.0	Straw.	Tr.	"	
	Diuresis.	2,725	75.0	0.8	1.8	20	8.0	Pink.	+++	"	
3	Control.	2,350	65.0	0.8	1.3	—	1.5	Straw.	0	Absent.	
	Diuresis.	2,450	73.0	0.8	1.6	20	8.0	Pink.	+++	"	
Dogs.	Time.										
1	—	7,250	72.0	1.6	5.8	—	2.5	Yellow.	0	"	
	Next day.			1.6	5.8	80	18.0	Orange-pink.	+++	Present.	
2	—	5,750	105.0	1.6	15.8	100	93.0	Pinky yellow.	+++	"	A rather dilute solution of hemoglobin was employed.
	2 days later.			1.6	15.8	—	15.5	Yellow.	0	Absent.	

3	—	7,250	73.0	1.8	9.6	40	22.0	Port wine.	+++	Present.
	7 days later. After 5 days more.		82.0	1.8	5.75	—	12.0	Yellow.	0	Absent.
				1.8	6.5	—	9.0	"	0	"
	After 3 days more.			1.8	6.5	100	64.0	Orange-pink.	+++	Present.

A different specimen of hemoglobin was used in this first observation from that employed in the three that followed.

½ gr. of morphine sulfate given prior to these observations.

The urines were all negative for hemoglobin prior to the injections; and at no time were red cells found.

test, and the injection made into an ear vein. A hemoglobin solution prepared by the method of Sellards and Minot[2] from the blood of rabbits was rapidly injected and followed in many instances by warmed 0.9 per cent salt solution. To rule out all possibility of a mistake in the composition of the latter solution such as might, by intravascular laking, cause hemoglobinuria, its effect was tested regularly on an erythrocyte suspension.

A table will be used to present the results (Table I). The amount of hemoglobin injected is there recorded in percentages of the total quantity of the pigment already in circulation, as calculated from the percentage in the blood (Palmer method) and the total blood quantity, assuming this to constitute 5.5 per cent of the body weight in the rabbit and 8 per cent in the dog. *Trial showed that in the rabbit one can inject hemoglobin up to at least 0.8 per cent of the body quantity without the appearance of any in the urine,* save when diuresis is induced. In the dog 1.6 to 1.8 per cent gives similar results. No attempt was made to determine the normal renal thresholds more closely. In each experiment with rabbits two individuals received the same preparation of hemoglobin in identical proportion, one animal serving as control while in the other diuresis was induced by the injection of 20 to 40 cc. of saline solution given over a period of several minutes. In the work with dogs, each animal served as its own control, receiving repeated injections.

Catheterization was done just prior to each injection and ½ hour after it. The guaiac test and the spectroscope were used to disclose hemoglobin, but in many instances more striking testimony was obtained in the bright pink or red hue of the urine. To determine whether hemorrhage had been a factor, erythrocytes and their shadows were sought for in the urinary sediment.

Nine pairs of rabbits were used, each pair closely matched as regards size and blood condition. In two pairs which received 0.6 per cent and 0.8 per cent of hemoglobin, the urine of all of the animals remained free from the pigment; but only a questionable diuresis had been induced. In a third pair diuresis yielded a red urine, whereas that from the control animal was pink; but both specimens showed fairly numerous erythrocytes. In the remaining six experiments diuresis regularly resulted in a pink or red urine containing much hemoglobin and free from red cells or shadows, in contrast to the specimens from the controls, which, though sometimes showing a few red cells, were always yellow and only once yielded a positive guaiac reaction after the cells had been removed. Three such sharp-cut instances are presented in the table.

Four dogs were used. In one the results were relative only, hemoglobin appearing in quantity when diuresis was induced, and to a less extent next day when diuresis was lacking after the pigment injection. The findings in the remaining three animals have been tabulated (Table I). It will be seen that diuresis regularly resulted in hemoglobinuria, whereas without it none occurred.

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[2] Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917–18, xxxvii, 161.

By varying the order of the experiment, an overloading of the organism with pigment was ruled out as responsible for the results.

It is evident that flood diuresis markedly influences the elimination of hemoglobin into the urine, lowering the renal threshold for the substance as it does that for dextrose. According to Cushny[3] flood diuresis probably never occurs in man as a result of fluid taken by mouth. The tubules of the rabbit kidney are much less active in resorption than those of human beings; yet when very large quantities of water are administered to this animal "even its feeble power of absorption is sufficient to save the optimal fluid."[3] The present findings, then, have no bearing on the occurrence of clinical hemoglobinuria. They are not without significance, however, for a proper understanding of the renal siderosis that occurs in diseases which involve the repeated liberation of small quantities of blood pigment into the circulation.

CONCLUSIONS.

Flood diuresis so far lowers the renal threshold for hemoglobin that the pigment appears in quantity in the urine as result of a hemoglobinemia insufficient under ordinary circumstances to lead to the elimination of even a trace of it. In pathological conditions that involve blood destruction hemoglobin probably passes into the tubules much more often than it reaches the urine, being prevented therefrom by the resorptive activity of the tubular epithelium.

[3] Cushny, A. R., The secretion of the urine, London, New York, Bombay, Calcutta, and Madras, 1917, 145.

SIGNIFICANCE OF THE HEMOSIDEROSIS OF PERNICIOUS ANEMIA. ·

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The view that pernicious anemia is due to an injurious agent derived from the gastrointestinal tract has of late years attained the proportions of a doctrine, and it has determined the direction of all recent efforts to find the cause of the disease. Whether such a view is inevitable is a question worth asking. In the present paper we shall show that one of the findings which has been considered well-nigh conclusive in its support is in reality worth little as evidence. We refer to the marked siderosis of the liver parenchyma that occurs during pernicious anemia, a localization of pigment which has been taken to indicate that pathological blood destruction is localized within the portal tributaries.

Hunter [1-6] in special has laid emphasis on the difference in distribution of the hemosiderin found in the organs of cases of pernicious anemia and that laid down as the result of frank blood destruction throughout the circulation as a whole. Siderosis in instances of the latter sort is usually far more marked in the kidneys and spleen than in the liver where it is especially abundant in pernicious anemia. Hunter's contention that a portal blood destruction is thus indicated in pernicious anemia receives support from the quite different distribution of the siderosis in animals in which hemolysis has been experimentally induced by injections into

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[1] Hunter, W., *Lancet*, 1888, ii, 556.

[2] Hunter, W., *Lancet*, 1888, ii, 608.

[3] Hunter, W., *Lancet*, 1888, ii, 654.

[4] Hunter, W., *Lancet*, 1903, i, 283.

[5] Hunter, W., *Lancet*, 1903, i, 367.

[6] Hunter, W., Pernicious anæmia: its pathology, septic origin, symptoms, diagnosis, and treatment, London, 1901.

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the systemic circulation[3, 6, 7, 8] and in those given intravenous or subcutaneous injections of hemoglobin. Schurig,[9] for example, found that daily injections of the hemoglobin of the horse into the subcutaneous tissue of rabbits led to an abundant siderosis of the kidneys with but a slight one of the liver.

Recent work on the removal of hemoglobin from the plasma by the liver and kidneys has suggested to us the possibility that differences in siderosis such as those described might be produced merely by varying the amount of pigment set free into the general circulation. We shall record experiments which prove that this is the case and then discuss the theoretical considerations leading to and deriving from them.

Method.

Young rabbits, mostly of less than 1,500 gm. weight have been used for the work. The liver of old normal animals of many species contains not infrequently a few granules of hemosiderin, but in immature rabbits of the size indicated they are not found. The hemoglobin was prepared from rabbit corpuscles by the method of Sellards and Minot.[10] It was given in concentrated watery solution of 120 to 160 per cent hemoglobin strength (Palmer) into the subcutaneous tissue of the flanks and abdomen, care being taken to select spots where there were no large vessels; and gentle massage was done to diffuse the pigment somewhat, but not greatly, since our aim was to bring about a gradual absorption throughout the 24 hours. We were able in many instances to assure ourselves that this actually happened, by direct inspection from time to time of the injection area through the translucent skin. Injections were given on 6 days out of every 7. For purposes of tabulation (Table I) the daily hemoglobin dose is expressed roughly, in terms of the total hemoglobin of the blood of each individual, as calculated from the hemoglobin percentage (Palmer), and the total blood quantity, assuming the latter to constitute 5.5 per cent of the gross body weight. There were no great differences in the hemoglobin percentages of the animals selected, so the only important variable was the weight. Many of the rabbits grew so rapidly as to necessitate frequent increases in the amount of pigment given. The injection period ranged from 13 to 102 days and the hemoglobin dose from $\frac{1}{4}$ that normally possessed by the animal to $\frac{1}{150}$ of it. The larger injections caused some loss of body weight, but in general the health of the animals was remarkably good.

[7] Hunter, W., Severest anæmias; their infective nature, diagnosis and treatment, London, 1909.

[8] Muir, R., and Dunn, J. S., *J. Path. and Bact.*, 1914–15, xix, 417.

[9] Schurig, *Arch. exp. Path. u. Pharmakol.*, 1898, xli, 29.

[10] Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917–18, xxxvii, 161.

The principal complication developing was a hemoglobinuria from the summated effect of the injections. Haessler [11] has noted that in the rabbit hemoglobinuria follows the rapid introduction into a vein of pigment amounts greater than $\frac{1}{125}$ of that possessed by the animal. Frequently in our experience, when a considerable amount of pigment had been introduced beneath the skin all was not absorbed before the next injection, and after a number of injections absorption would be going on from several different regions at once with result that pigment appeared in the urine. When small amounts were used, only a slight brownish stain could be detected in the subcutaneous tissue after 24 hours, yet even in such instances the urine tests were often eventually positive for hemoglobin. Whether this was due to an overtasking of the abilities of the other organs removing pigment from the blood, or resulted from changed local conditions we have not inquired. Induration at the site of injection rarely occurred.

The urine of most of our rabbits was daily submitted to the guaiac test, though, as is well known, a positive reaction does not always mean hemoglobinuria in these animals, and we were but seldom able to demonstrate blood pigment with the spectroscope. That it was actually present in the urine in many of the positive instances was indicated by their time of occurrence, which was after several large injections or a considerable number of small ones.

When large amounts of hemoglobin had been given, the renal cortex proved to be snuff-colored from siderosis, but the color of the liver was never distinctly altered from the normal, and the spleen was quite unenlarged and unchanged in hue. Chloroform was the routine lethal agent. Blocks from the liver, kidney, spleen, and red bone marrow were fixed in alcohol and stained with ammonium sulfide, and in duplicate, by the combined ammonium sulfide-potassium ferrocyanide method of Nishimura. Muir and Dunn[8] advocate the use of hot hydrochloric acid in carrying out Nishimura's technique, and, confirming their results, we find that iron is much more sharply demonstrated thereby. But since practically all of the data on the distribution of hemosiderin in human beings have been obtained by the less perfect methods above mentioned, we have given most attention to their use. The criterion used in gauging the degree of siderosis was likewise that employed with most of these data, namely the appearance of the tissue under the microscope. Since only special cells and regions of each organ undergo pigmentation, a decision as to which organ contains most hemosiderin cannot be reached, unless the differences are pronounced.

RESULTS.

The distribution of hemosiderin, as ascertained by the means described, differed markedly with the amount of hemoglobin given (Table I). When a daily portion of less than $\frac{1}{50}$ of the approximate quantity of pigment in circulation was employed, practically no

[11] Haessler, H., *J. Exp. Med.*, 1922, xxxv, 515.

TABLE I.

Relative Siderosis of the Rabbit Liver and Kidneys.

Rabbit No.	Final weight.	Proportion of animal's own hemoglobin given each time	Period covered by injections	Observed siderosis.	Remarks.
	gm.		*days*		
1	1,750	$\frac{1}{150}$	80	None.	No tests made for hemoglobinuria.
2	1,850	$\frac{1}{120}$	80	"	No tests made for hemoglobinuria.
3	1,600	$\frac{1}{120}$	37	Very slight; more in liver than kidneys.	Guaiac test occasionally positive; spectroscopic findings negative.
4	1,800	$\frac{1}{100}$	102	None.	No tests made for hemoglobinuria.
5	2,100	$\frac{1}{100}$	68	"	No tests made for hemoglobinuria.
6	1,550	$\frac{1}{90}$	37	Much of liver; none of kidneys.	Guaiac test occasionally positive; spectroscopic findings negative.
7	1,650	$\frac{1}{80}$	72	Much of liver; a few granules in kidneys.	No tests made for hemoglobinuria.
8	1,500	$\frac{1}{75}$	37	Much of liver; some of kidneys.	Old scarring of kidneys in both instances. Guaiac test occasionally positive; spectroscopic findings negative.
9	1,650	$\frac{1}{60}$	37	Much of liver; some of kidneys.	
10	1,650	$\frac{1}{30}$	20	Marked kidney siderosis; little of liver.	No hemoglobin ever in urine.
11	1,375	$\frac{1}{30}$	19	Similar amount in both organs.	Hemoglobin demonstrated in urine with the spectroscope.
12	1,350	$\frac{1}{20}$	18	Much kidney siderosis; little of liver.	Hemoglobin found once with the spectroscope.
13	2,000	$\frac{1}{8}$	13	Very marked kidney siderosis; little of liver.	Hemoglobin often in the urine as shown with the spectroscope.
14	1,750	$\frac{1}{4}$	13	Very marked kidney siderosis; little of liver.	Hemoglobin almost regularly in the urine.

siderosis was anywhere to be found, even after the injections had been kept up for many weeks. By the use of hot hydrochloric acid a blue dot could sometimes be demonstrated within the nucleus of many cells of the convoluted tubules of the kidneys, but with the ordinary technique it was not evident and so may be dismissed from attention. After slightly larger injections, long continued, the liver parenchyma exhibited a well defined stippling with hemosiderin, which, like that of pernicious anemia, was most pronounced near the periphery of the lobules; whereas the kidneys were non-pigmented, or negligibly so. With still more hemoglobin, the differences in the organs became less noteworthy and sometimes the kidney tubules showed an equal or greater siderosis. Always when large injections had been given, resulting in hemoglobinuria after but a few days, the epithelium of the renal tubules was heavily siderosed with coarse granules or lumps, and the hepatic parenchyma was by contrast negligibly pigmented. Often in such instances an amorphous material giving the iron reaction was to be seen lying free in the glomerular capsule and the lumen of the upper tubules, a material such as Muir and Dunn[8,12] noted after intravascular hemolysis. In the spleen no special siderosis was ever found, thus bearing out Schurig's contention that this organ, while a great locus of deposit for injured cells, has no special power to retain the pigment derived from them. The same would seem to be true of the bone marrow, to judge from our specimens.

An attempt was made to repeat the work with dogs, but several obstacles intervened that proved decisive. Dog hemoglobin has a marked tendency to crystallize out of solution. We were unable to keep it for more than a few hours in concentrations greater than 55 per cent (Palmer); and to administer the necessary amounts in dilute form entailed large injections that were often ill-borne. Furthermore, the relative vascularity of the subcutaneous tissue of the dog, as compared with that of the rabbit, and the wide extension of the injected material through it, combined to result in such rapid absorption that hemoglobinuria was rendered frequent. On the other hand, when the pigment had not been spread by massage, abscesses often developed. In the dog, too, the Kupffer cells have what would seem

[12] Muir, R., and Dunn, J. S., *J. Path. and Bact.*, 1915–16, xx, 41.

to be an unique predilection for hemoglobin. In several of our animals the liver sections submitted to the ferrocyanide reaction, appeared deep blue to the unaided eye, obviously containing enormous quantities of iron, but with the microscope this iron was seen to be confined almost wholly to the Kupffer cells which showed both a granular and a diffuse coloration. The six young dogs of our experiments were given hemoglobin in amounts of from $\frac{1}{80}$ to $\frac{1}{180}$ of that possessed in the blood. All ultimately developed hemoglobinuria and were sacrificed for that reason. The findings as regards relative siderosis of the liver and kidney parenchyma were inconclusive, as might have been predicted from these facts.

The renal siderosis in both the dog and rabbit affected especially the cells of the proximal convoluted tubules and of the ascending limb of Henle's loop, as is the case in pernicious anemia, but not in hemochromatosis,[13] a disease in which there is no evidence of pathological blood destruction.

<center>DISCUSSION.</center>

We have shown that the constant presence in the general circulation, as distinct from the specifically portal, of a small amount of free hemoglobin leads eventually to a siderosis of the liver similar to that which has been considered so significant in pernicious anemia. When this amount is kept within certain limits, renal siderosis fails to appear or is negligible in degree. When more is given, the epithelium of the renal tubules rapidly becomes pigmented, the iron deposition far outstripping that in the liver. These are the facts. The conception which led to the experiments demonstrating them,— and which itself receives strong support from them,—will now be outlined. It is based on the differing activities of the liver and kidneys in the elimination of hemoglobin.

The liver possesses a special ability to remove free hemoglobin from the blood stream,[14] and under normal circumstances is probably the principal organ, if not the only one, that keeps the plasma free from the pigment. It may still succeed in so doing,—and in the

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[13] Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913-14, vii, 129.

[14] Dubin, H., and Pearce, R. M., *J. Exp. Med.*, 1917, xxv, 675.

doing become siderosed—when there is a persistent slight increase in the amount of hemoglobin coming to it.   Its ability, though, is over-tasked by any considerable frank hemolysis,—as is often evidenced by the hemoglobinuria then ensuing; and when this is the case, the pigment accumulates in greater or less quantity in the blood, to be dealt with by the kidneys according to the laws governing the fate of threshold substances.   Hemoglobin is, like dextrose and many foreign dyestuffs, a threshold substance in its renal relations,[15] one which readily passes the glomerular barrier but undergoes a greater or less resorption during its passage through the tubules.   One would suppose, *a priori*, that hastening this passage would favor hemo-globinuria by lessening the opportunities for resorption, just as glycosuria is favored.   And, indeed, Haessler,[11] in a paper from this laboratory, has demonstrated the fact that flood diuresis causes marked hemoglobinuria, when it is induced immediately after the intravenous injection of an amount of blood pigment so small that none passes into the urine under ordinary circumstances.   All this being true, it is obvious that there must be many clinical occasions when hemoglobin passes into the tubules but fails to reach the urine, owing to the completeness of its resorption.   A similar resorption of foreign dyestuffs leads to their deposition in quantity in the tubule cells.   Does not renal siderosis come about in a like manner?   And, when there is much blood pigment to be resorbed, will not the unusual opportunities of the tubule cells to obtain it lead to a noteworthy hemosiderin deposition within them?   The results of our experiments indicate that these questions are to be answered in the affirmative. The findings do not permit of a conclusion as to whether the freedom of the kidney from hemosiderin when small amounts of hemoglobin have been administered is due to a glomerular threshold for the latter substance, as distinct from the greater renal one.   Not infrequently, after large injections of hemoglobin, the amorphous contents of the glomerular capsule and tubules yields a positive reaction for iron, a fact which strongly suggests that hemosiderin may circulate as such in the blood and be excreted in the urine where indeed one of us has already found it lying free in granular form.[16]

[15] Cushny, A. R., The secretion of the urine, London, New York, Bombay, Calcutta, and Madras, 1917.

[16] Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.

TABLE II.

Relative Siderosis of the Liver and Kidney in Pernicious Anemia, Determined Microscopically.[*]

Author and reference.	No of cases.	Degree of siderosis.		Technique used.
		Liver.	Kidney.	
Quincke, H., *Samml. klin. Vortr.*, 1876, No. 100,797.	2 1	Equal in both.		
		Marked.	Very slight.	
Quincke, H., *Deutsch. Arch. klin. Med.*, 1877, xx, 1.	1	"	None.	Potassium ferrocyanide and hydrochloric acid.
Quincke, H., *Deutsch. Arch. klin. Med.*, 1879–80, xxv, 567.	2 2	" "	Slight. None.	Ammonium sulfide.
Mott, F. W., *Lancet*, 1889, i, 520.	1	"	"	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
Mott, F. W., *Lancet*, 1890, i, 287; *Practitioner*, 1890, xlv, 81.	1	"	Slight.	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
Griffith, J. P. C., and Burr, C., *Tr. Assn. Am. Phys.*, 1891, vi, 239.	1	" "	" None.	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
Hopkins, F. G., *Guy's Hosp. Rep.*, 1893–94, l, 349.	5	Equal in both.		Potassium ferrocyanide and hydrochloric acid.
Stühlen, A., *Deutsch. Arch. klin. Med.*, 1894–95, liv, 248.	1 3 2	" Marked. Moderate.	" Slight. None.	
Warthin, A. S., *Am. J. Med. Sc.*, 1902, cxxiv, 674.	4 4	Equal in both. Marked.	Less.	Technique not mentioned.
Hunter, W., *Lancet*, 1903, i, 367.	1 1	Slight. Moderate.	Moderate. Slight.	Potassium ferrocyanide and hydrochloric acid.

[*] The reports of Hunter's many cases [6,7] do not lend themselves well to tabulation.

TABLE II—*Concluded.*

Author and reference.	No. of cases.	Degree of siderosis.		Technique used.
		Liver.	Kidney.	
Gulland, C. L., and Goodall, A., *J. Path. and Bact.*, 1905, x, 125.	3	Equal in both.		Potassium ferrocyanide and hydrochloric acid.
	5	Marked.	Moderate.	
	3	"	Slight.	
	4	Present.	None.	
	2	"		
Schneider, J. P., *J. Am. Med. Assn.*, 1920, lxxiv, 1759.	5	Equal in both.		Technique not mentioned.
	5	Marked.	Moderate.	
	2	"	None.	

The observed variations in the distribution of hemosiderin in pernicious anemia are rendered understandable by the facts just presented. In Table II a summary is given of the records of several observers on siderosis in the disease. It will be seen that while an hepatic pigmentation not infrequently exists without any siderosis of the kidney, and is usually relatively marked when the latter occurs, yet cases are not wanting in which the renal siderosis equalled that in the liver (Table II). Such individual differences in the siderosis of the disease may be due to the same cause that has yielded like findings in the animals of our experiments, *viz.* differences in the amount of free hemoglobin in the general circulation. Livers that are damaged—and the organ is assuredly damaged in pernicious anemia as a fatty change attests—not infrequently become somewhat siderosed in the absence of a pathological blood destruction;[17] and when this is present pigmentation may become extreme.[18]

Let it be granted, despite the foregoing, that blood destruction during pernicious anemia is mainly portal. Even this constitutes no reason for the belief that the disease has its seat within the viscera draining to the liver by the portal stream. Too little notice has been taken of the circumstance that red cells, damaged in many body regions and in many different ways, are alike "scrapped"

[17] Kretz, R., *Beitr. klin. Med. u. Chir.*, 1896, No. 15.
[18] Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

within the spleen, whence the liberated hemoglobin passes to the liver. Thus it is with cells damaged by a burn of the skin,[19] by a hemotoxin,[20] or specific hemolysin injected into a peripheral vein, by chemical agents such as toluylenediamine,[21] or by the principles responsible for incompatibility on transfusion. Even under normal conditions, the same local deposition of corpuscular debris goes on,[22] as it does also to a much more considerable extent during repair from secondary anemia, when the unusually frail cells put forth by the marrow are threshed to pieces whilst in circulation.[23] All this is to say that many sorts of blood destruction which are systemic in origin become portal in completion. So it may well be with the destruction that takes place during pernicious anemia.

A brief comment upon the other evidence for a portal origin of pernicious anemia will not be out of place. Stress has been laid upon the resemblance between the disease and *Bothriocephalus* anemia, and on the results of experiments whereby a blood picture not dissimilar from that of pernicious anemia has been produced through the action of hemolytic substances absorbed from the gastrointestinal tract. There can be, of course, no doubt that substances affecting a liberation of hemoglobin within the portal vessels will cause a more distinctive liver siderosis than those active within the circulation generally. Yet the latter can suffice as we have shown. The symptoms from the alimentary tract during pernicious anemia, and the anatomical changes ultimately found therein, constitute no stronger evidence on the seat of the disease than do the nervous symptoms and changes. The most that can be said of them is that they are suggestive. But no matter how suggestive they seem in association with the other facts just mentioned, it is surely good policy to box the compass of possibilities in the consideration of so obscure a disease as pernicious anemia, rather than to look for enlightenment in a fixed direction.

[19] Askanazy, M., in Aschoff, L., Pathologische Anatomie, Jena, 4th edition, 1919, i, 69.

[20] For example, the megatheriolysin of Todd.

[21] Joannovics, G., Z. *Heilk.*, 1904, xxv, 25.

[22] Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.

[23] Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665.

SUMMARY.

The selective deposition of hemosiderin in the liver parenchyma during pernicious anemia does not constitute evidence that there is a hemolytic cause for the disease located in the portal region. The repeated introduction of small amounts of free hemoglobin into the general circulation, by the subcutaneous route, leads, as we have shown, to an identical siderosis. Larger amounts of hemoglobin cause a renal pigmentation equalling or exceeding the hepatic, a fact that is in keeping with what is known of the physiology of hemoglobin excretion and of the findings in human beings after outspoken hemolysis.

THE RENAL ELIMINATION OF BILIRUBIN.

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The mode of escape through the kidneys of circulating blood and bile pigment has received but scanty attention in the past as compared with that of foreign dyestuffs. These last have been the subject of a multitude of experiments and of controversies that are not yet at an end. The reason for this is plain. The dyestuffs have been utilized for the better discovery of principles which would apply supposedly to the body pigments as well. But since these principles are still ill defined and their general application is unattested, direct studies on the renal elimination of hemoglobin and bilirubin would seem desirable—more especially since patients with these pigments in their urine continue to demand attention.

Authorities agree that the kidney is often severely injured by jaundice. Albuminuria and cylindruria (Nothnagel) appear early. Quincke[1] has comprehensively described the anatomical changes. At an early stage the cortex is diffusely stained with bilirubin. As time passes the pigment accumulates in granular form in the cells of the convoluted tubules and more markedly in those of the loop of Henle, and in the lumen of the latter many free granules may be seen, together with yellow, green, or brown casts. It is noteworthy that the glomeruli remain practically unstained. There is cloudy swelling of the tubular epithelium, with loss of the brush border, and even necrosis here and there. In Quincke's opinion, these severe changes cannot but result in a lessened renal activity, and thus may have serious consequences for the organism as a whole.

Significance of Jaundiced Cells in the Urine.

According to text-books on clinical microscopy a "bile-stained urinary sediment" is a regular accompaniment of marked jaundice. It has been tacitly assumed that the bile staining is a staining of dead

[1] Quincke, H., in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1899, xviii, 63.

cells by the fluid in which they live immersed, and is devoid, therefore, of clinical significance. Such is often the case. But there is another possibility, that some of the cells may be kidney cells impregnated with pigment prior to desquamation into the urine. Such cells would afford a direct index to the renal condition, like the cells containing granular hemosiderin that are to be found in the urine of patients with hemochromatosis and pernicious anemia.[2]

In the urine of icterus neonatorum, cells containing granular and crystalline bilirubin are almost regularly present, often indeed when no dissolved pigment can be demonstrated. An excellent paper on the theme is that of Cruse[3] whose technique for the microscopic Gmelin reaction we have found highly useful. No systematic observations have been reported on the urinary cells of jaundiced adults, or at least none carried out from the standpoint of kidney physiology.

We have studied the urinary sediment of many icteric human beings and of dogs with jaundice produced in several ways—by fasting, by blood destruction with toluylenediamine or a specific hemolytic serum, and by ligation of the common duct. Two types of cell staining with bilirubin have been discriminated, one consequent on sojourn of the elements in the urine, the other a direct expression of the renal condition. In the one the cells from all parts of the urinary tract are stained, whereas in the other, no matter how deep the jaundice, the leucocytes, squamous epithelium, and bladder epithelium are uncolored, whereas the cells of manifest renal origin may be deeply stained. Needless to say, both types of staining, or only that first mentioned, will be seen in urines that have stood for a long time. The specific pigmentation of the renal cells is most evident in specimens freshly voided; and some hours are required for the bilirubin to dissolve out of the more heavily impregnated cells. After long continued jaundice in man, the urinary sediment yields striking indications of the serious condition of the kidneys. Numerous cells, often disintegrating, will be observed, crowded with coarse, opaque, brown granules or irregular particles, and many such particles lie free. All give under the microscope an intense Gmelin reaction. The pigment cannot be confused with the yellowish lipoidal substances

[2] Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.

[3] Cruse, P., *Arch. Kinderheilk.*, 1880, i, 353.

found in some pathological states.[4]   Intracellular clumps of narrow
brown needles are frequently present.   The cytoplasm of the renal
cells is usually stained deep yellow, though not always.   The des-
quamated elements from the lower urinary tract are by contrast
practically colorless, though lying in a dark, icteric fluid.

Special interest attaches to the findings in subicteric states and
during slight and transient jaundice.   Bile pigment is a threshold
substance in human beings, one which readily passes into the tubules
but is absorbed again in its course through them, and so rapidly that
often none can be found in the urine when a considerable quantity
exists in the blood.   Will the cast off renal cells yield evidence of this
absorption process when the plasma contains bile pigment but tests
fail to disclose it in the urine?   Such has not proved to be the case.
In urines that fail to give the Gmelin reaction, cells stained with
bilirubin are absent.   Evidently there must be in human beings a
special glomerular threshold for bilirubin as well as a' more general,
higher one for the kidney as a whole.   For were this not so, the renal
epithelium found in the urine should be tinted with bilirubin when-
ever the plasma is colored with it—and the plasma is so colored
normally.

Human urine during slight or transient jaundice regularly con-
tains renal cells tinted a diffuse yellow and yielding the Gmelin reac-
tion, in contrast to the colorless and negatively reacting elements of
the lower tract.   Granular bilirubin is not seen.   We are inclined
to believe that Rosenbach's method of test,[5] whereby much urine is
passed through a filter and this latter submitted to the Gmelin reac-
tion owes its delicacy, in part at least, to the accumulation upon the
paper of specifically stained cells.

In dogs the slightest and most transient jaundice may lead to an
output of renal cells brilliantly stippled with bilirubin.   The pigment
occurs as small or coarse, rounded or oblong, granules of a bright
mahogany-brown, scattered irregularly in the ground glass cytoplasm
of large cells with a rather small, rounded nucleus.   The cytoplasm
itself is usually unstained save sometimes for a very distinct brownish
red zone, or halo, around each granule.   In kept specimens such

[4] Weicksel, J., *Deutsch. Arch. klin. Med.*, 1919, cxxx, 260.
[5] Rosenbach, O., *Centr. med. Wissensch.*, 1876, xiv, 5.

halos of dissolved bilirubin regularly develop. These and the ruddy tint of the pigment go far to differentiate the latter from hemosiderin in the absence of chemical tests. When the urine stands for 24 hours at room temperature, the brown granulation usually disappears but in the ice box it persists much longer. Severe jaundice is accompanied in both dogs and rabbits by heavily granulated cells and free particles of bilirubin in the urine, just as in the case of human beings.

It is a curious fact that many dogs with slight jaundice yield only diffusely tinted renal cells, whereas in others with no greater icterus, elements stippled with bilirubin are regularly encountered. This difference is consistently maintained over considerable periods of time. We have noted it day after day in catheterized specimens from animals possessing normal kidneys, and with "physiological icterus" induced by fasting. It is independent of the reaction of the urine or of diuresis. Quite possibly the difference is one in derivation of the cells, those from the stippled portions of the tubules failing to desquamate in some animals.

Effects of Flood Diuresis in Dogs.

The sources of damage to the kidneys during jaundice have never been precisely determined. The excretion of bile salts may be, and probably is, far more injurious than that of bile pigment, yet there is no doubt that the accumulation of the latter in the renal cortex, as in the system generally, should be avoided if possible. Diuresis has long been advocated for the purpose. We have tested out its efficacy upon dogs.

Animals were selected that during a period of several days showed no albuminuria or casts. They were kept in metabolism cages. Under ether, all of the large bile ducts were separately ligated, or the common duct was tied and cut and the neck of the gall bladder similarly obstructed to rule out any influence of this reservoir upon the course of the jaundice. Asepsis was maintained throughout, and the incision was closed in three layers. The accumulation of bilirubin in the blood, together with its output in the 24 hour urine, was carefully followed by means of the quantitative method of van der Bergh and Snapper[6] for the blood, and Hooper and Whipple's[7] modification of the Salkowski method for the urine.

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[6] van der Bergh, A. A. H., and Snapper, J., *Deutsch. Arch. klin. Med.*, 1913, cx, 540.

[7] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

TABLE I.

Effect of Flood Diuresis on Bilirubinuria.

Time of occurrence after operation.	Time.	Procedure.	Fluid injected.			Urine.			Bilirubin units.			Blood.	
days			Total. (cc.)	Rate per kilo per min. (cc.)	Period. (hrs.)	Total. (cc.)	Output per hr. (cc.)	Color.	Output per cc.	Total.	Output per hr.	Hemoglobin. (per cent)	Bilirubin content per 100 cc. of plasma. (mg.)
18	9.00 *a.m.*	Catheterized.											
19	10.00				24	100	4.17	Dark brown.	100	10,000	417	89	1.47
	11.15	Catheterized. Injection begun.		0.37	2¼	27	12.0	"	89	2,400	1,067		
	11.45	Speed of injection altered.	100	0.17									
	2.15 *p.m.*	Catheterized. Speed of injection altered.	325	0.185	3	23	7.7	Orange.	91	2,090	698		
	3.00		400										
	3.30	Catheterized.	500	0.37	1¼	45	36.0	Yellow.	58	2,610	2,090		
	4.00	Injection stopped.	550	0.185									
	4.15	Catheterized.			¾	40	53.3	"	40	1,600	2,133	87	1.33
20	9.00 *a.m.*				16¾	325	19.4	"	42	13,630	814		
21	9.00				24	150	6.25	Light brown.	100	15,000	625	87	1.72

After 10 or more days of obstruction had elapsed and the icterus had reached a relatively constant level, the study of the effects of intravenous injections of fluid was begun. The animal was stretched out; the bladder was emptied with a catheter; and warmed 0.9 per cent sodium chloride solution was introduced into
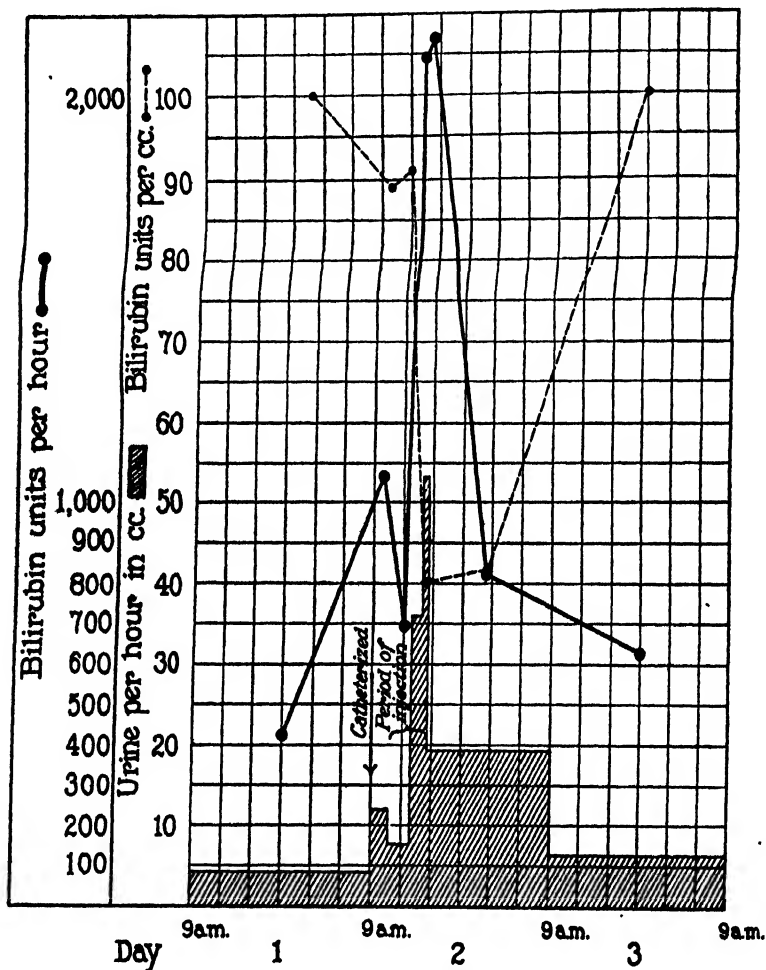
Text-Fig. 1. Effect of flood diuresis on bilirubinuria.

the saphenous vein at a measured rate of from 0.17 to 0.7 cc. per kilo per minute. Dastre and Loye[8] have stated that even with the maximum rate here given no fluid accumulates in the tissues or blood; and we have assured ourselves that this holds true for the blood by taking its hemoglobin content from time to time during

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[8] Dastre and Loye, P., *Arch. physiol. norm. et path.*, 1888, ii, series 4, 93.

the experiment. The injection was continued for 3 hours or more, during which time the urine was collected by catheter and its pigment content determined. The experiment was successfully carried out four times in all, on three dogs.

The findings were consistent. The diuresis consequent on the injection of salt solution regularly increased the output of bile pigment greatly. The amount per cubic centimeter of urine, on the other hand, was much lessened, unlike that of hemoglobin, which under similar circumstances[9] is notably augmented. The intensity of the bilirubinemia remained practically unaffected. A single protocol will suffice to show the findings.

Experiment 1.—A brown female collie weighing 9 kilos was operated upon 19 days before the diuresis experiment and all duct branches were tied, and, where possible, cut. The amount of bilirubin contained in the urine was not measured in milligrams but in terms of an arbitrary standard of units, so called. The findings for this reason are without quantitative significance in relation to the bilirubin content of the blood. Table I gives the course and results of the experiment and Text-fig. 1 illustrates it graphically.

Effects in Dogs of Diuresis from Water by Mouth.

The effects of large amounts of water administered by mouth to dogs with the jaundice of total obstruction were followed in several series of animals.

Dogs tolerate total biliary obstruction for many weeks but lose appetite, drink little, and gradually emaciate and become anemic. The kidneys undergo a progressive injury. For these reasons it was deemed best to begin the observations within a few days after the ducts had been obstructed at operation, and by alternating periods of forced fluid by mouth with those in which there was only the normal intake to render each animal its own control. Dogs with normal blood and urine were selected for the work. Since the character and amount of the food are supposed to influence the quantity of bile pigment formed[10] a constant ration of bread and meat was supplied each day and the amount taken was determined. Fortunately the animals ate about as much when water was forced as during the control periods.

Much difficulty was experienced in determining the precise amount of pigment eliminated in the urine from day to day, more especially during the periods of diuresis. The following modification of Hooper and Whipple's method was

[9] Haessler, H., *J. Exp. Med.*, 1921, xxxv, 515.

[10] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

finally employed. 2 per cent of the 24 hour specimen was made up to 20 cc. with water, alkalinized with sodium carbonate, the bilirubin precipitated out with calcium chloride as usual, and the bluish green coloration obtained with acid alcohol was read in a colorimeter against the color got through the action of the same kind of alcohol on a chloroform solution of bilirubin (Schuchardt) containing 1 mg. in every 4 cc.[11]

The early results indicated that not only was the bilirubin output not increased during several successive days of diuresis, but that toward the end of the period it fell almost to zero. Since such a finding is contrary to all that is known of the effects of diuresis on renal elimination, control tests were undertaken.

Various mixtures of icteric and non-icteric urines, some of them concentrated, some dilute as the result of diuresis, showed that the difficulty did not lie with the method itself, which quantitated bilirubin equally well, no matter how dilute the urine.

Further controls, involving variations in the amount of sodium carbonate used in the alkalization of the urine, and in the concentration of acid in the acid alcohol used to redissolve the precipitate, proved that these factors had no essential influence to cause error. Incidentally, it may be remarked that precipitated calcium bilirubinate was found to keep well in the ice box, and the precipitate from our specimens was sometimes kept for 24 to 48 hours prior to quantitation.

At length, tests were made to determine the value of cage as compared with catheterized specimens. The observations indicating a decreased bilirubin output during diuresis had been made upon urine collected in a vessel placed beneath the metabolism cage in which the animal was kept. Such urine was only protected from fecal contamination by a coarse grating in the bottom of the cage. The animal during non-diuresis periods drank very little water and almost invariably passed a dry formed stool, so that during these periods the amount of fecal contamination of the urine was slight. During periods of forced diuresis, on the other hand, a loose watery stool was frequently passed, and the urine specimens then contained large amounts of fecal material. Our practice had been to filter the mixed total urine for the 24 hour period and make tests on the filtered specimen. Parallel tests now made on filtered and unfiltered specimens disclosed considerable differences in bilirubin content (Table II). What is more, it was found that the sediment of a centrifuged cage specimen, though twice washed with distilled water, still may contain as much as four times the amount of bilirubin present in the urine from which it had been separated.

Since the animals serving as our example (Table II) had complete biliary obstruction, as subsequently determined at autopsy, while, furthermore, repeated

[11] McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1921, xxxiii, 731.

tests of their stools when unexposed to urine invariably gave negative reactions for bilirubin, the fecal matter can be excluded as the original source of the pigment.

We believe that these findings point to a precipitation of bilirubin out of the urine by some of the fecal elements. They have been set forth at length because it is the accepted practice to use cage urine for bilirubin estimations.

TABLE II.

Effect of Fecal Contamination on the Quantitation of Bilirubin in the Urine.

Dog.	Urine specimen.	Total 24 hr. output of bilirubin as calculated from the filtered and unfiltered specimens.		Remarks.
		Filtered.	Unfiltered.	
		mg.	*mg.*	
1 Male mongrel; weight 5 kg.	1	19	18	Practically no feces present.
	2	13	13	" " " "
	3	8	23	Marked fecal contamination.
	4	6	14	" " "
	5	11	19	" " "
	6	11	16	" " "
	7	6	13	" " "
2 Male mongrel; weight 6 kg.	1	9	13	Marked fecal contamination.
	2	4	5	" " "
3 Male mongrel; weight 8 kg.	1	5	6	Slight fecal contamination.
	2	9	8	" " "

It has been usually assumed that in the intestinal canal bilirubin is transformed to urobilin and related substances by the action of bacteria. We have observed that when bile-containing urine is mixed with bile-free fecal matter and incubated at 37°C. for 24 hours, a large proportion of its bilirubin is destroyed, while the remainder, no longer in solution, is to be found with the fecal sediment.

Following the recognition of these sources of error, some further carefully controlled experiments were made upon the effects of diuresis in jaundiced dogs.

TABLE III.

Effect on Bilirubin Excretion of Diuresis from Water by Mouth.

Period.	Day.	Duration of excretion.	Amount.	Output per hr.	Urine. Type of specimen.	Bilirubin content.	Bilirubin output per hr.	Blood. Hemoglobin.	Blood. Bilirubin content per 100 cc. of plasma.	Water by gavage.	Remarks.
		hrs.	*cc.*	*cc.*		*mg.*	*mg.*	*per cent*	*mg.*	*cc.*	
First non-diuresis period.	1	6	72	12.0	Catheterized.	5.61	0.93	69	4.04		
		18	115	6.4	" and voided.	16.56	0.92				
		Total or average.	187	7.8		22.17	0.92				
	2	6	35	5.8	Catheterized.	7.10	1.18	69	4.06		Weight 5.2 kg.
		18	91	5.0	" and voided.	21.20	1.18				
		Total or average	126	5.2		28.30	1.18				
	3	6	42	7.0	Catheterized.	5.41	0.90	72	4.92		
		18	109	6.1	" and voided.	17.36	0.96				
		Total or average.	151	6.3		22.77	0.95				
	4	6	24	4.0	Catheterized.	4.83	0.80	72	4.78		
		18	70	3.9	" and voided.	15.80	0.88				
		Total or average.	94	3.9		20.63	0.86				

Weight 5 kg.

5	24	100	4 2	Voided.	27.20	1.13	72	5.90	
6	6	25	4 2	Catheterized.	5.00	0.83			500
	18	67	3.7	" and voided.	19.40	1.08			
	Total or average.	92	3 8		24 40	1 02			
7	6	33	5 5	Catheterized.	6.43	1.07	66		
	18	178	9 9	"	17.71	0 99			
	Total or average.	211	8 8		24.14	1.00			
8	6	750	125 0	Catheterized.	7.73	1.29	59	3.7	750
	18	133	7 4	" and voided.	15.11	0.84			
	Total or average.	883	36 8		22 84	0.95			
9	6	579	96.0	Catheterized.	7.10	1.18	72	1.5	750
	18	439	24.4	" and voided.	15.12	0.84			
	Total or average.	1,018	42.5		22.22	0.93			
10	6	495	82.5	Catheterized.	6.54	1.09	62	4.04	1,000
	18	195	10.8	" and voided.	15.22	0.85			
	Total or average.	690	28.7		21.76	0.91			

First diuresis period.

TABLE III—*Concluded.*

Period.	Day.	Duration of excretion.	Urine.					Blood.		Water by gavage.	Remarks.
			Amount.	Output per hr.	Type of specimen.	Bilirubin content.	Bilirubin output per hr.	Hemoglobin.	Bilirubin content per 100 cc. of plasma.		
		hrs.	*cc.*	*cc.*		*mg.*	*mg.*	*per cent*	*mg.*	*cc.*	
Second non-diuresis period.	11	24	250	10.4	Voided.	15.40	0.64	57	5.03		Weight 5 kg.
	12	24	405	16.9	Voided.	14.13	0.59				
	13	24	260	10.8	Voided.	9.60	0.40	56	5.30		
Second diuresis period.	14	24	885	36.8	Voided.	14.60	0.61	56	5.30	750	
	15	6	399	66.5	Catheterized.	6.27	1.04	49	4.60	750	
		18	352	19.5	" and voided.	13.50	0.75				
		Total or average.	751	31.3		19.77	0.82				
	16	6	320	53.4	Catheterized.	6.11	1.02	43	2.45	1,000	
		18	530	29.4	" and voided.	12.76	0.71				
		Total or average.	850	35.4		18.87	0.79				

| 17	6 / 18	688 / 153	114.8 / 8.5	Catheterized. / " and voided.	6.11 / 13.79	1.02 / 0.77	43	1.95	800	Weight 4.2 kg.
	Total or average.	841	35.1		19.90	0.83				
18	24	109	4.5	Voided.	18.80	0.78	43	2.3		
19	24	144	6.0	Voided.	23.20	0.97	45	4.0		
20	24	132	5.5	Voided.	16 40	0.68				
21	24	232	9.7	Voided	27.8	1.16	45	4.0		

Third non-diuresis period.

Thymol was added to the urines to check bacterial action; unfiltered specimens were employed, with their proportionate share of fecal sediment when this latter was present; and as much as possible of the 24 hour output was obtained by catheterization. Precipitation with calcium chloride was carried out on each specimen immediately after it had been procured. The bilirubin output was
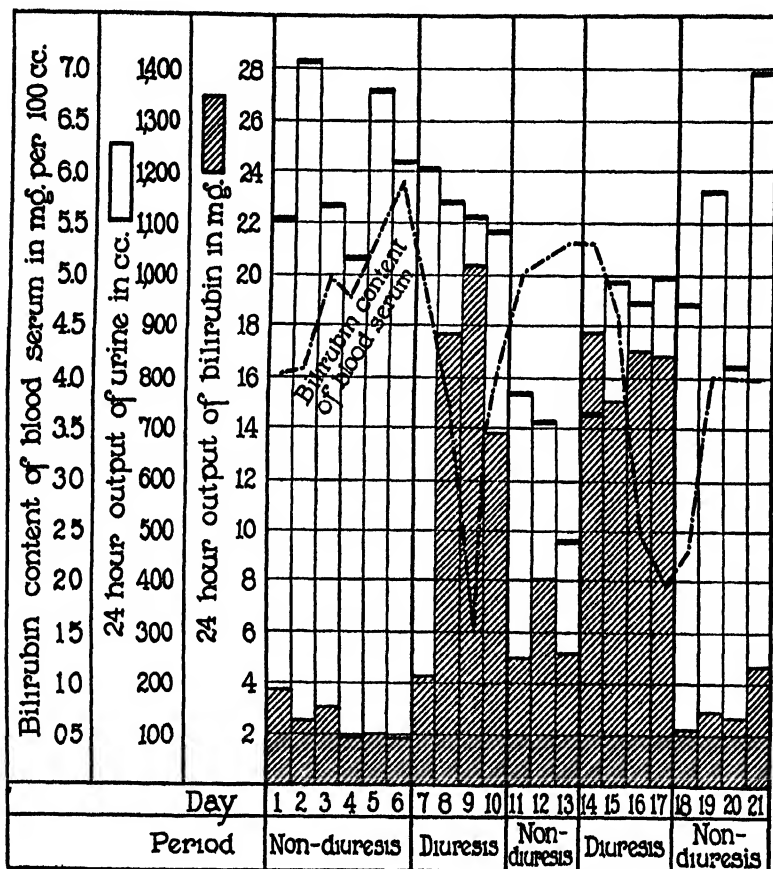
TEXT-FIG. 2. Effect on bilirubin excretion in the urine of diuresis from water by mouth.

quantitated during alternate periods in one of which no effort was made to increase the water intake, which was invariably low, while in the other water was given by stomach tube, usually in 250 cc. amounts at 2 hour intervals three times each day.

A type instance of the results of the experiments is recorded in Table III and portrayed in Text-figs. 2 and 3. It will be noted that

Text-Fig. 3. The same as Text-fig. 2 except that the results are presented in greater detail.
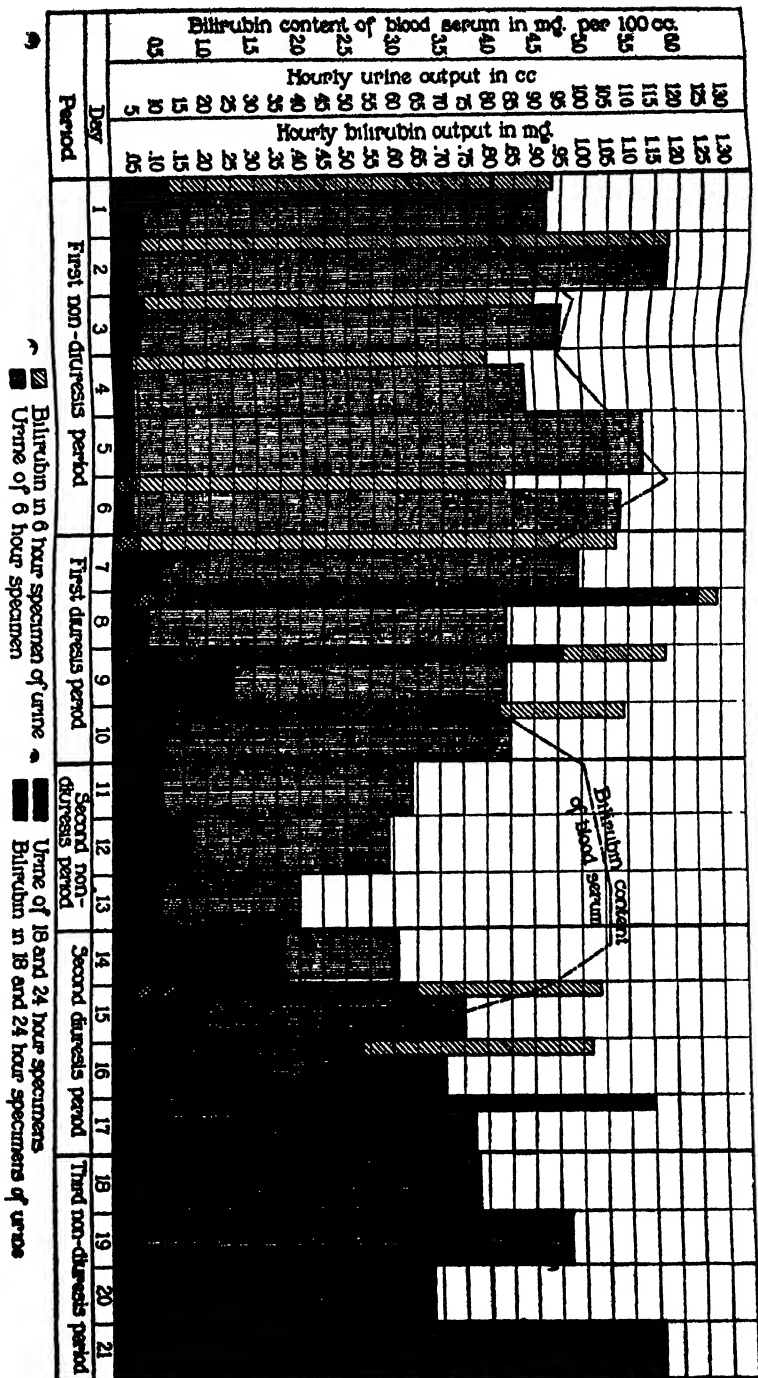
on the 1st day when water was given by stomach tube, practically no diuresis ensued, owing probably to the fact that the animal had drunk but little for some days previously and fluid was needed by the tissues. A similar result was also obtained in later observations on diuresis in jaundiced human beings. On all subsequent days in which water was given there occurred marked diuresis, but as the greater part of the water was eliminated within 2 hours after each administration of it, there was only about 6 hours of diuresis altogether in each 24. The average urinary output for the remaining 18 hours was but slightly greater than that during the control, non-diuresis, periods. Cushny[12] has brought evidence that flood diuresis, such as follows upon intravenous injections, is practically never caused by fluid absorbed from the gastrointestinal canal, and we must assume that it had not occurred in our animals, despite the rapidity of the fluid elimination.

It will be seen that the bilirubin output per hour is fairly regular throughout each day of the first or non-diuresis period, the hourly average between 10 a.m. and 4 p.m. being in general somewhat lower than during the remaining 18 hours. Unfortunately no figures are available for the hourly output between 10 a.m. and 4 p.m. in the second and third non-diuresis periods. Only the average output per hour for the 24 hour period was recorded. In both of the diuresis periods, as already remarked, the increase in quantity of the urine was limited practically to the period between 10 a.m. and 4 p.m. The hourly output of bilirubin during this time is distinctly greater than during the remainder of the day. On the other hand, the output in the remaining 18 hours, when the urine was relatively scanty, is decreased to such extent that the output for the entire 24 hours averaged a little less than that during the first and third non-diuresis periods. True, the average 24 hour output for the second non-diuresis period is the lowest of the series, but there is no evidence to show that this was due to the administration of water during the preceding days. The main point sought after is clearly shown; diuresis from water by mouth has remarkably little effect on the day to day elimination of bilirubin.

[12] Cushny, A. R., The secretion of the urine, London, New York, Bombay, Calcutta, and Madras, 1917, 136.

The bilirubin of the blood plasma was followed by means of the diazo reaction, as has already been stated.   There occurred a fall in the pigment concentration during the diuresis periods, but this can be explained in several ways other than by increased elimination of bilirubin from the body, notably by the accumulation of fluid in the tissues.   Both in dogs and in human beings a fluid retention was indicated by changes in the weight.   Quite possibly less bile pigment was produced in the organism during the periods of diuresis than in the intervals between.

Effects of Diuresis in Jaundiced Patients.

Observations were made on two men with catarrhal jaundice in whom periods of diuresis from forced fluid by mouth were alternated with periods of restricted water intake.[13]   The icterus was marked when the observations were begun, but thereafter gradually and regularly diminished.   The marked variations that were induced in the daily output of urine through forcing water by mouth had no evident effect on the rate at which the jaundice lessened from day to day as determined by the diazo reaction on the blood plasma.   And the content of the 24 hour urine in bilirubin was no greater when the voidings amounted to several liters than when only half of this quantity had been passed.   The findings, then, confirming those in dogs, show that diuresis by alimentary means fails, practically speaking, to increase bilirubin elimination.

DISCUSSION.

The foregoing experiments offer little support for the view advanced by some clinicians that forcing water by mouth has a direct effect to diminish the intensity of jaundice, by increasing the elimination of bilirubin in the urine.   The flood diuresis which follows an intravenous injection of salt solution brings out, it is true, a relatively considerable quantity of bile pigment, but the method is clinically inapplicable, and, as has been shown, a copious diuresis from water, by mouth, yields no such consequence, so far as the 24 hour output is concerned.   The fact is well recognized that "all the constituents

[13] These observations were made through the courtesy of Dr. E. F. Du Bois.

of the urine are increased in absolute amount per unit of time during diuresis."[12] In the present instance, the increase is more important theoretically than actually. Yet obviously this need not mean that diuresis is valueless as a means wherewith to combat the effects of bile retention. Its influence on the output of bile salts—substances more injurious to the organism than the pigments—remains to be determined when proper methods become available. Furthermore, diuresis may help to avert the accumulation of bilirubin in the kidneys with the disordered function consequent thereon.

Nonnenbruch[14] has shown that the acutely disordered kidney may fail to eliminate bile pigment during jaundice. It is probable that the extraordinarily pronounced icterus seen in some human beings during the later weeks of total biliary obstruction is due in part at least to a lessening of renal elimination. We have obtained data which would seem to bear significantly upon this point. In the attempt to increase jaundice in dogs, some injections of hemoglobin were given intravenously to animals already the subject of a long standing, total biliary obstruction and the mild general icterus that this entails. Hemoglobin for the purpose was obtained by the method of Sellards and Minot;[15] and eight to ten successive hourly injections were given, of amounts slightly less than that which should, on calculation, cause hemoglobinuria. In this way it proved possible to intensify the icterus markedly. At the end of a day of injections, the scleras of the dogs were pronouncedly more yellow than at the beginning. But the icterus was not maintained. By next morning only the previous, relatively pale, coloration characteristic of total obstruction was present. The urine during the transition period was heavily loaded with bilirubin. It is probable that in the case of bilirubin, as of urea, any increase in the circulating amount beyond a certain point is compensated for, when the kidneys are normal, by an increase in ease of elimination.

In man, as already stated, bilirubin is a threshold substance, in its renal relations, and it is normally present in appreciable amount in the blood. But in the dog, not only is the blood normally free from it, but whenever bilirubinemia can be detected there is bilirubinuria,

[14] Nonnenbruch, W., *Mitt. Grenzgeb. Med. u. Chir.*, 1918–19, xxxi, 470.

[15] Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917–18, xxxvii, 161.

while often the latter is to be found alone.[11] The question arises whether actually there is no threshold for bilirubin in this species, or whether the current tests for bilirubinemia are at fault. The presence in freshly voided urine, during even the slightest bilirubinuria, of renal cells specifically stained or stippled with the bile pigment constitutes evidence in this connection. According to the modern view enunciated by Cushny, all substances that pass into the urine leave the circulation by way of the glomerulus, and the so called threshold substances undergo resorption to a greater or less degree during their passage through the tubules. Thus it is that when the threshold substance is a dyestuff, the tubular epithelium becomes pigmented. Were all this quite certain, the existence in the urine of the dog of jaundiced kidney cells would be proof that in

TABLE IV.

Maximal Bilirubin Output during Total Obstruction.

Dog No.	Body weight.	Bilirubin.		Time of occurrence after operation.
		Expected 24 hr. output.	Greatest actual 24 hr. output.	
	kg.	*mg.*	*mg.*	*days*
1	4½	39.6	19.5	28
2	5½	45.8	28.3	28
3	9½	81.4	24.0	10

this species bile pigment is a threshold substance. But as happens, there is recent work to show that some, at least, of the tubules have an excretory function.[16] Therefore, our cellular evidence is unconvincing.

The amount of bile pigment eliminated in the urine of the dog during complete and long continued biliary obstruction is never nearly so great as that formed during the same period by a normal liver. The bilirubin output of fistula animals in good condition amounts to about 8.8 mg. per kilo of body weight per day.[7] Table IV shows the observed pigment content of the urine of three dogs with persistent total obstruction. It will be seen that the maximum output of Dog 3 occurred on the 10th day of obstruction and that

[16] Oliver, J., *J. Exp. Med.*, 1921, xxxiii, 177.

of the other two animals on the 28th day.   In all three instances the bilirubin content of the blood had already become fairly constant, and so too had the tissue icterus.   The 24 hour output of pigment in the urine never approached the amount which a normal liver would have secreted in the same time.   Several explanations suggest themselves for the discrepancy, more especially diminished liver function during jaundice, and destruction of pigment within the body.

SUMMARY.

The elimination of bile pigment during jaundice is, for practical purposes, unincreased by diuresis from water by mouth.   Possibly, though, the flushing of the kidneys tends to lessen pigment accumulation within these organs and thus to diminish a serious potential source of trouble in long continued jaundice.   Flood diuresis from intravenous injections of salt solution markedly increases the output of bile pigment.   It is important to know the effect of variations in the urinary output on the elimination of bile salts, but methods for the purpose are not available at present.

The passage of bile pigment into the kidney cells during jaundice is attested by the presence in the freshly voided urine of desquamated renal elements specifically stained, stippled, or granulated with bilirubin.   Pigmentation of this sort is readily to be distinguished from the indiscriminate staining of cellular debris that occurs in icteric urines on standing.   It has clinical significance, furnishing direct·evidence on the degree of renal change.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VII. SEROLOGICAL REACTIONS.

BY PETER K. OLITSKY, M.D., AND FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 37.

During the fall and winter of 1918–19, and the early spring of 1920, we collected a number of specimens of blood serum from patients in the active stages of epidemic influenza, or after recovery from the disease, for the purpose of studying the reactions of the sera with strains of *Bacterium pneumosintes*[1] which had been isolated from the nasopharyngeal secretions of influenza patients and from the lung tissues of rabbits inoculated with these secretions. Samples of blood serum were collected also from rabbits which had been allowed to recover after showing the characteristic clinical picture produced by intratracheal injections of the active nasopharyngeal secretions, or had been experimentally inoculated with the sediment from tissue cultures of *Bacterium pneumosintes*.

The results of our efforts to demonstrate specific antibodies in these serum specimens were disappointing. The sparse growths of the organism in the earlier generations, mixed with the protein precipitate that develops in the Smith-Noguchi medium, did not provide an antigen suitable for serological tests. On account of non-specific precipitation and concomitant sedimentation, precipitin and agglutinin tests were unsatisfactory and indeterminate. Therefore, at the time the sera of influenza patients and of most of the affected rabbits were available we were unable to make use of them for lack of a suitable antigen.

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[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

More recently a method has been developed by one of us (Gates) by which certain pathogenic anaerobes, including *Bacterium pneumosintes*, may be cultivated in a collodion sac dialysate of the Smith-Noguchi medium.[2] The ascitic fluid or dilute serum and the fresh tissue fragment are placed within the sac, which is surrounded by distilled water or physiological salt solution under a vaseline seal. In the course of 24 to 48 hours anaerobic conditions are established throughout the system and the nutritive and growth-promoting substances in the tissue medium have diffused through the membrane in sufficient quantities to support growth in the surrounding liquid, while the protein precipitate that collects around the tissue fragment is retained within the sac.

Bacterium pneumosintes grows readily in this anaerobic tissue culture dialysate, visibly clouding the clear liquid in a few hours and producing a heavy turbidity in 3 to 5 days. When growth is checked through exhaustion of the nutritive material or the accumulation of deleterious substances the organisms gradually settle out of suspension, leaving a clear supernatant fluid over a compact, slightly brownish sediment. Films of these cultures show only the stained organisms, without the background of precipitate that is deposited by the tissue medium.

Preparation of Immune Serum.

When it was possible to cultivate *Bacterium pneumosintes* by this method in quantities sufficient for use, two rabbits were injected intravenously with doses of the living culture of Strain 11. This strain had been recovered from the lung tissue of a rabbit representing the eighth animal passage of active material derived from the nasopharyngeal secretions of Patient 11[3] of the 1918–19 epidemic. During immunization one rabbit (A) developed secondary infections and was killed. The other rabbit (B) received five injections of 2 to 4 cc. of a thin suspension of live culture at intervals of 5 to 7 days and was bled on the 9th day thereafter. The sterile serum was stored at 4°C. without preservative. In the first series of tests with

[2] Gates, F. L., *J. Exp. Med.*, 1922, xxxv (in press).

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

this serum it was examined for the presence of agglutinins, precipitins, bacteriotropins, and complement-fixing bodies against two strains of *Bacterium pneumosintes*, No. 11, the homologous strain, and No. 26, from the recurrent epidemic of 1920.

Since then the serum of this rabbit and similar sera produced in five other rabbits have been studied in various serological reactions with four strains of *Bacterium pneumosintes*, three (Nos. 11, 16, and 17) from the first epidemic (1918–19), and one (No. 26) from the second (1920). The results of the latter experiments are all in accord with those of the first series and need not be reported in detail. The following tests are described to indicate the methods and results of the first serological experiments.

Agglutination.

Agglutination tests were first made with a mixture of live organisms and undiluted immune serum from Rabbit B spread on a cover-slip, sealed with vaseline over a hollow slide, and examined microscopically immediately or after 30 minutes incubation at 37°C. Both strains tested (Nos. 11 and 26) were promptly agglutinated, with the formation of large compact masses of organisms in a clear surrounding fluid (Fig. 1). Control tests with normal rabbit serum showed no agglutination, or only the occasional clumping of three or four bacteria.

Macroscopic agglutination tests were then set up to determine the titer of the immune serum. Measured drops of serum dilutions 1:5 to 1:160 were mixed with equal quantities of bacterial suspension and sealed in capillary tubes of 2 mm. internal diameter.[4] After incubation for 2 hours at 37°C. the tubes were placed in the ice box over night and read the next morning. Strain 11 was completely agglutinated in a serum dilution of 1:80, with the limit of visible agglutination in a serum dilution of 1:320. The corresponding limits for Strain 26 were 1:40 and 1:160, respectively. Control tests with normal rabbit serum showed no agglutination even in a serum dilution of 1:2. The contrast in the behavior of Strain 11 in immune and in normal serum is shown in Fig. 2.

Precipitation.

Precipitin tests were also performed in glass tubes of 2 mm. bore, in which the antigen and the serum were carefully layered by drawing them up in succession by means of a rubber bulb. The antigen used was the clear supernatant fluid from old cultures of *Bacterium pneumosintes* grown in the dialysate of a tissue medium. After incubation for 1 hour at 37°C. a sharp opaque line of precipitation

[4] Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvii, 2054.

was observed at the juncture of the antigen and the immune serum from Rabbit B. In undiluted, normal rabbit serum a doubtful line of precipitation was observed with Strain 11 only. The appearance of the experimental and control tubes is shown in Fig. 3.

Complement Fixation.

The antigens used in the complement fixation test were the same as those for the precipitation reaction. Preliminary control tests showed that these antigens were not hemolytic or anticomplementary in volumes of 0.5 cc. when tested with 0.1 cc. of 40 per cent guinea pig complement and 2 units of anti-human amboceptor against 0.1 cc. of a 10 per cent suspension of human red cells. The immune serum of Rabbit B and the control normal serum were not hemolytic or anticomplementary in dilutions of 1:5. Antigen 0.45 cc., complement 0.1 cc., and the test sera in dilutions up to 1:160, made up to a volume of 1.05 cc. with physiological saline solution, were incubated together in a water bath at 37°C. for 30 minutes. After the addition of 0.1 cc. of corpuscle suspension and 0.1 cc. (2 units) of dilute amboceptor the tubes were again incubated for 30 minutes at 37°C. Complement fixation was complete with both antigens (Strains 11 and 26) in the highest dilution of immune serum tested; namely, 1:160. No fixation occurred in the tubes containing the normal rabbit serum.

Phagocytosis.

In the phagocytic experiments the Neufeld method[5] was used to test for the presence of bacteriotropic substances. Rabbit leucocytes were obtained from subcutaneous tubes[6] containing aleuronat in agar. Large mononuclear cells (monocytes) and lymphocytes, as well as polynuclear neutrophils, collect in these tubes and may be observed in phagocytic experiments. In the tests to be described the diluted leucocytic suspensions were mixed with suspensions of young dialysate cultures of *Bacterium pneumosintes* in physiological salt solution, or in dilutions of normal or immune rabbit serum. The mixtures were incubated 1 to 4 hours at 37°C., films prepared, fixed in methyl alcohol or by heat, and stained with Löffler's alkaline methylene blue, Wright's blood stain, or Cross' stain for leucocytes.[7] In salt solution or in normal serum controls only an occasional leucocyte picked up a few organisms. Unphagocytosed bacteria were plentiful and unagglutinated. In the presence of immune serum from Rabbit B, however, two phenomena occurred. Especially in low dilutions of the immune serum the bacteria were gathered into clumps. Leucocytes had attacked and

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[5] Neufeld, F., and Rimpau, W., *Deutsch. med. Woch.*, 1904, xxx, 1458; *Centr. Bakt., 1te Abt., Ref.*, 1906, xxxvii, 763.

[6] Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 280.

[7] Cross, H. B., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 51.

sometimes surrounded these clumps, with which they were engorged. Other leucocytes had engulfed single organisms and contained from a few to very many of them. Both the polynuclear cells and the large mononuclear cells (monocytes) were actively phagocytic. Small mononuclear cells (lymphocytes) did not engulf the bacteria. The highest serum dilution in which an increased phagocytic activity was clearly evident was 1:80. Photographs of these phases of the reaction are shown in Figs. 4 to 7.

In addition to this series of experiments with the serum of Rabbit B and Strains 11 and 26 of *Bacterium pneumosintes*, we have made serological tests with Strains 16 and 17 and with the serum of five other rabbits immunized by repeated injections of these strains. Sera produced with certain strains, when tested in cross-agglutination experiments with the other strains, have agglutinated them and the homologous organisms in practically the same dilutions. No specific differences among the three strains from the 1918–19 epidemic or between any of these and Strain 26 from the recurrent epidemic of 1920 have been found, and the·serological evidence indicates their antigenic identity. This is what might be expected if they were all derived from a common source.

It is evident from the results of these experiments that the reaction of the animal body to *Bacterium pneumosintes* involves the production of the antibodies which are commonly recognized by serological tests. We have no reason to suppose, therefore, that the mechanism of protection against this organism, of which we have evidence in the immunity reactions already described,[3] differs from that which comes into play in the case of infections with aerobic pathogenic organisms.

We would have welcomed the opportunity of testing the sera of influenza patients during the recent epidemic, or of rabbits experimentally infected with nasopharyngeal secretions from these patients. · At first a suitable antigen was lacking. More recently almost all of the glycerolated material on hand was rendered inactive through the inadvertent use of glycerol which was later found to be chemically impure. The following single experiment, therefore, represents our only opportunity to test against *Bacterium pneumosintes* the sera of rabbits which had been subjected to the active agent in glycerolated form.

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

*Specific Agglutinins in the Blood of Rabbits after the Injection of Glycero-
lated Material.*

September 12, 1921.   Two rabbits were injected intratracheally with glycero-
lated lung tissue from a previously injected rabbit which had showed the typical
clinical and pathological picture already described.[3]   This animal represented
the seventh passage of the active agent derived from Case 26.[3]   The two injected
rabbits in turn showed conjunctivitis, an increase in temperature, and the char-
acteristic fall in the total leucocyte count due to a drop in mononuclear cells.
Both were allowed to recover, and were bled from the ear vein for a serum sample

Serum dilutions													
Serum	Strain	1 2	1 4	1 8	1 16	1 32	Serum	Strain	1 2	1 4	1 8	1 16	1 32
Immune rabbit B	16	+++	+++	+++	+++	+++	Normal rabbit 3	16	−	−	−	−	−
	17	+++	+++	+++	+++	++		17	−	−	−	−	−
	26	+++	+++	+++	+++	++		26	−	−	−	−	−
Experimental rabbit 1	16	+++	++	+	−	−	Normal rabbit 4	16	−	−	−	−	−
	17	+++	++	+	−	−		17	−	−	−	−	−
	26	++	+	−	−	−		26	−	−	−	−	−
Experimental rabbit 2	16	++	+	−	−	−	Normal rabbit 5	16	−	−	−	−	−
	17	++	+	+	−	−		17	−	−	−	−	−
	26	+	+	−	−	−		26	−	−	−	−	−

Text-Fig. 1. Agglutinins in the serum of rabbits injected with active glycero-
lated lung tissue.

on the 21st day after injection.   These sera together with three normal rabbit
sera and the immune serum from Rabbit B were then tested for agglutinins
against killed, washed, dialysate cultures of *Bacterium pneumosintes* (Strains 16,
17, and 26).   The results of this experiment are shown in Text-fig. 1.

This experiment shows that the animal body reacts to experimental
infection with the active agent of the nasopharyngeal secretions of
influenza patients by antibody formation against *Bacterium pneumo-
sintes*, and completes the proof of the identity of the active agent
with the organism obtained from the same immediate and original
sources.

SUMMARY.

Cultivation of *Bacterium pneumosintes* in the collodion sac dialysate of a tissue medium produces an antigen suitable for serological tests.

Injection of dialysate cultures of *Bacterium pneumosintes* into rabbits results in the production of antibodies demonstrable by agglutination, precipitation, complement fixation, and phagocytic reactions.

Four strains of *Bacterium pneumosintes*, three from the first epidemic influenzal wave (1918–19) and one from the second (1920), show identical antigenic characters.

The blood serum of rabbits experimentally injected with the glycerolated active material of rabbit passages contains specific agglutinins for *Bacterium pneumosintes*, whereas normal rabbit serum does not.

EXPLANATION OF PLATE 37.

Figs. 1 and 2. Agglutination of *Bacterium pneumosintes* in immune serum from Rabbit B.

Fig. 1. Edge of hanging drop, dried and stained.   × 15.

Fig 2. Macroscopic agglutination in capillary tubes.   *A*, immune serum, complete agglutination; *B*, normal rabbit serum, no agglutination. The white crescent at the bottom of each column is not sediment but light reflected from the meniscus.   × 1

Fig 3 Precipitation reaction with the supernatant fluid of *Bacterium pneumosintes* cultures *versus* rabbit serum.   *A*, Strain 11 and immune serum from Rabbit B; *B*, Strain 11 and normal rabbit serum; *C*, Strain 26 and immune serum from Rabbit B; *D*, Strain 26 and normal rabbit serum.   × 1.
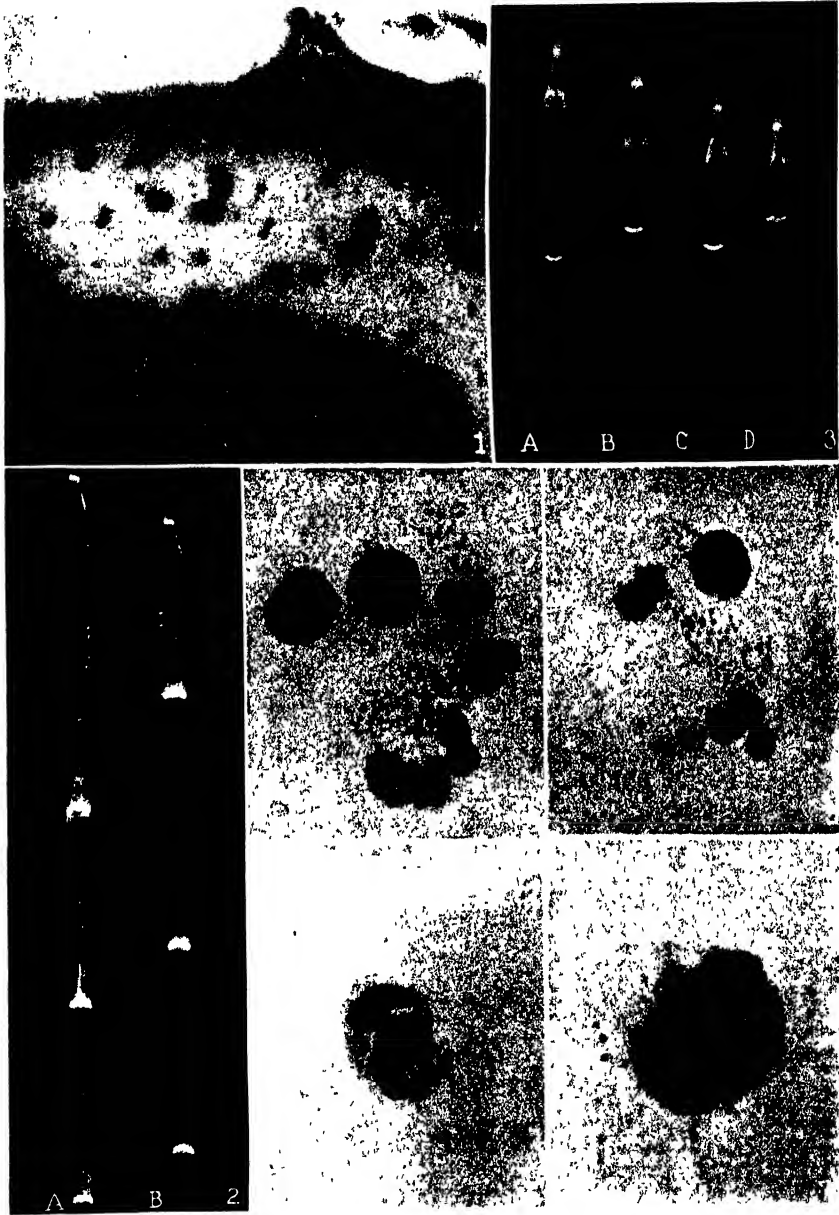
Figs 4 to 7.   Phagocytosis of *Bacterium pneumosintes* in immune serum from Rabbit B.

Figs. 4 and 5.   Monocytes (transitional cells) attacking agglutinated groups of organisms.   × 1,000.

Fig. 6. Polynuclear neutrophil containing organisms.   × 1,100.

Fig. 7. Monocyte containing organisms.   × 1,500.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VIII. Further Observations on the Cultural and Morphological Characters of Bacterium pneumosintes.

By PETER K. OLITSKY, M.D., and FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 67.

In an earlier paper of this series[1] *Bacterium pneumosintes*, derived from the nasopharyngeal washings of patients in the early hours of acute epidemic influenza, was described as a minute bacilloid body of regular form, with a length about two to three times its breadth, measuring 0.15 to 0.3 micron in the long axis. It was stated that although longer individuals were seen occasionally, "the organisms showed little tendency to pleomorphism and were characterized by uniformity in size and shape."

At that time, *Bacterium pneumosintes* had been cultivated only in a tissue medium composed of human ascitic fluid and fresh rabbit kidney, sometimes with the addition of beef infusion broth and nutrient agar. The combination of ascitic fluid and fresh kidney tissue has remained the medium of choice for the maintenance of the cultures, because in it they require transfer only at long intervals and they retain their morphological and cultural characteristics through many generations. But during cultivation over a period of 1 to 3 years the three strains[2] at present available have become saprophytic, so that at present they are cultivable anaerobically in a variety of media which are less difficult to prepare. Coincident with this adaptation to a new environment, certain variations in morphology and a loss of pathogenicity for rabbits have been observed. It is the

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[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

[2] These strains were derived from Cases 16, 17, and 26 (Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125).

purpose of this paper to describe the cultural and morphological characteristics of *Bacterium pneumosintes* after prolonged artificial cultivation. The three strains have behaved in an identical manner, and a common description will suffice.

Methods of Cultivation.

Even after cultivation for 3 years *in vitro Bacterium pneumosintes* fails to grow in autoclaved media without the nutritive or growth-stimulating factors found in fresh tissue or body fluids. But the ascitic fluid of the original medium may be replaced by peptone broth; and fresh defibrinated blood, vegetable tissue,[3] or the products of bacterial metabolism may be substituted for rabbit kidney. The addition of dextrose (1 per cent) to the medium hastens the establishment of anaerobic conditions, and, judging from the rapidity and density of the growth, increases its nutrient value.

The basis of the media now in use is a 1 per cent peptone beef infusion broth, with sodium chloride 0.5 per cent, titrated to pH = 7.4 on the Sörensen scale. According to the object for which the culture is prepared, this broth is enriched with fresh blood, animal or vegetable tissue, or by the growth of other organisms, as will be described. Solid and semisolid media are made by the addition of nutrient agar in the proper proportion.

When a large inoculum from an enriched medium is added to dextrose broth alone, *Bacterium pneumosintes* grows in the primary, but not in subsequent transplants, showing that the initial growth is dependent upon factors carried over from the tissue medium. Thjötta and Avery[4] have shown how minute is the quantity of the growth-stimulating factors required in the case of Pfeiffer's bacillus. Their V factor in dilutions of 10^{-3} and their X factor in dilutions of 10^{-6} suffice to promote growth. With *Bacterium pneumosintes* the

[3] Dr. O. T. Avery and Dr. H. J. Morgan, at the Hospital of The Rockefeller Institute, found that *Bacterium pneumosintes* grows readily in a simple infusion broth containing a fragment of fresh vegetable tissue (potato, turnip, parsnip, etc.) and kindly permit us to report their observation in advance of their own publication. They described this medium in the *Proceedings of the Society for Experimental Biology and Medicine*, 1921, xix, 113.

[4] Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 97.

essential growth-stimulating factors appear to be of the same order of efficacy. For example, 20 mg. of fresh rabbit kidney supports growth in 5 cc. of medium. The faint haze of *Bacillus coli* which develops in the 1st hour after inoculation makes dextrose broth a highly favorable medium for *Bacterium pneumosintes*. This interesting symbiotic relationship will be discussed in a subsequent section.

Cultural Characteristics.

Bacterium pneumosintes was described as an obligate anaerobe and has maintained that character. We have successfully cultivated the organism only under a vaseline seal or in the depths of the medium in the presence of active reducing agents such as dextrose, fresh animal or vegetable tissue, or aerobic organisms, or in a strictly anaerobic jar. Under such conditions, under a vaseline seal for example, *Bacterium pneumosintes* grows in enriched dextrose broth in a diffuse cloud throughout the fluid medium. In previously incubated tubes, in which anaerobic conditions are already established, this cloud becomes visible within a few (8 to 16) hours after inoculation and reaches its greatest density in 3 to 5 days, when the culture is opaque by reflected light and shows a smoky translucency by transmitted light. Growth is then checked by acid production. The cloud is too finely divided to show a bacterial shimmer, and remains in suspension for days, gradually settling in an even, amorphous, cream-colored layer in the bottom of the tube. Spontaneous flocculation has not been observed.

In the depths of solid media submicroscopic colonies develop as tiny gray specks, which, under the high power of the microscope, are found to be dense irregular masses of bacteria with a fringe of single organisms.

Dextrose is split by *Bacterium pneumosintes* with acid formation but without gas production. In dextrose broth the limiting hydrogen ion concentration is the same for all three strains; namely, 5.2 to 5.3 on the Sörensen scale. After growth has apparently ceased, however, the organisms remain viable for several days in the acid medium.

Recently the simple, safe, and efficient anaerobic jar described by Brown[5] has furnished us with the conditions necessary for the

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[5] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

development of surface colonies of *Bacterium pneumosintes*. The jar is a modification of those devised by McIntosh and Fildes[6] and by Smillie,[7] and utilizes the catalytic activity of palladinized asbestos heated by a resistance coil in a wire-screened chamber (the principle of the Davy safety lamp) for the union of the oxygen with hydrogen. In this jar we have used plates and slants of nutrient agar, made with beef infusion broth without dextrose, enriched with 5 per cent of fresh defibrinated rabbit blood.

After incubation for 7 to 10 days blood agar plates sown with *Bacterium pneumosintes* show many very minute colonies, almost submicroscopic in size, which are round, raised, and convex, with an entire edge and a colorless translucency. No characteristic structure has been observed, and the growth does not discolor or precipitate the medium. On account of their minute size the colonies are usually discrete, even when close together, but they coalesce to form raised plaques of confluent growth in the most crowded areas.

These colonies interest us as the first example of which we are aware of surface colony formation by a filter-passing obligate anaerobe. The plates also give us a ready means of purifying contaminated cultures, and are useful for the demonstration of organisms in sparse growths of early generations in the ascitic fluid-kidney medium in which the microscopic observation of *Bacterium pneumosintes* is difficult on account of its minute size and the presence of stained protein precipitate.

Morphology.

The substitution of dextrose-peptone broth for ascitic fluid or serum in the medium results in a considerable change in the morphology of *Bacterium pneumosintes*. The dextrose, by the prompt establishment of anaerobic conditions and by its nutritive value to the organisms, supports a much more luxuriant growth than is found in ascitic fluid media. The bacteria are found in diplo form, or in chains of several members, and many of the individual organisms have increased in length so as to be obviously bacillary. They are plump rods with rather pointed ends, which give them a spindle

[6] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

[7] Smillie, W. G., *J. Exp. Med.*, 1917, xxvi, 59.

shape. Stains color them deeply only in the middle and fade out towards the ends. In chains the spaces between the members are sharply demarcated. More variation occurs in length than in thickness. Forms from 0.5 to 1.0 micron long are not uncommon in cultures which show the characteristic minute forms also. Figs. 1 and 2 show the relative size and shape of *Bacterium pneumosintes* in a collodion sac dialysate of ascitic fluid and in dextrose broth respectively. A strain which has grown as fusiform bacilli in dextrose broth for several generations reverts to the original minute form on cultivation in a dialysate of the original ascitic fluid medium (Fig. 3). Aside from the differences in length and the greater tendency to chain formation, no irregularities in morphology have been noted in comparison with the early generations.

In films from surface colonies grown for 7 days on rabbit blood agar in an anaerobic jar, most of the organisms are of the minute form, but here also longer individuals are found.

As stated previously, *Bacterium pneumosintes* decolorizes by Gram's method. The organisms stain with the usual basic dyes. They are not motile. Neither capsules nor flagella have been demonstrated.

Serological Reactions.

The fusiform, bacillary forms of *Bacterium pneumosintes* grown anaerobically in enriched dextrose broth media and on blood agar plates have been tested at intervals with the immune rabbit sera produced a year ago with cultures of the organism grown in the collodion sac dialysate of an ascitic fluid-rabbit kidney medium.[3] All three strains are promptly agglutinated by these sera, and, on the other hand, they show no tendency to spontaneous flocculation or to agglutination in normal serum. Their genetic relationship to the original minute forms of *Bacterium pneumosintes* is without question, and is further evidenced by their strictly anaerobic character and their reversion to the minute forms on transfer to the original medium.

Symbiosis.

Mention has already been made of the symbiotic development of *Bacterium pneumosintes* with *Bacillus pfeifferi*, the pneumococcus,

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

Streptococcus hæmolyticus and *viridans*, and staphylococci, in cultures accidentally contaminated with these organisms.[1] In a number of instances *Bacterium pneumosintes* was recovered from these mixed cultures by filtration. Since the medium then in use was sufficient for the independent growth of any of these organisms, the coincident development of *Bacterium pneumosintes* and the contaminating organism did not give evidence of a nutritive interchange between them.

More recently, in cultures intentionally inoculated with a strain of *Bacillus mesentericus*, we have observed the growth of *Bacterium pneumosintes* in autoclaved dextrose broth in the absence of a fresh tissue fragment. This observation has led to the development of a simple method of anaerobic cultivation. Aerated dextrose broth is inoculated with a young culture of *Bacillus mesentericus* in which spores have not yet formed. During the first hours after inoculation the organism grows diffusely in a faint cloud without pellicle formation. The reducing activity of this obligate aerobe is shown by the decolorization of methylene blue in such a culture within a few minutes after its introduction. The culture is sealed with vaseline when inoculated, or shortly afterward and growth is inhibited by the exhaustion of free oxygen from the medium. Meanwhile the hydrogen ion concentration of the medium falls from 7.4 to 7.2–6.8. 6.8 appears to be the limiting acidity for our strain of *Bacillus mesentericus*. If such a preparation is then inoculated with *Bacterium pneumosintes*, the anaerobe grows luxuriantly and clouds the medium heavily in the course of 1 or 2 days.

This rapid growth of *Bacterium pneumosintes* carries the hydrogen ion concentration of the medium to 5.3–5.2 in the course of 3 or 4 days. Coincidently the vegetative forms of *Bacillus mesentericus* are killed and undergo autolysis so that finally microscopic films and plate cultures may show only the anaerobic organisms. Usually, however, some few spores of *Bacillus mesentericus* are introduced in the original inoculum or are formed in the culture. These may develop subsequently and complicate results. We have therefore tested a number of other organisms for their nutritive or growth-promoting properties, combined with other characters which make them suitable for use. Of these, *Bacillus coli communis* seems well adapted to our purpose. It multiplies rapidly in .plain or dextrose

broth under a vaseline seal, so that a sufficient growth is obtained within an hour or two after inoculation. During this short incubation the hydrogen ion concentration of the medium is only slightly affected. The organism may then be killed by exposure to 100°C. for 15 to 30 minutes without destroying the peculiar nutritive substances it imparts to the medium. At the same time anaerobic conditions are immediately established under the vaseline seal. *Bacillus coli* is practically non-pathogenic, and its autolyzed products are not toxic in the minute amounts contained in these cultures. The *Bacillus coli* broth is inexpensive and simple to prepare, and less liable to contamination than are unheated media containing fresh tissue or blood. It contains a minimum of foreign protein. Finally, it supports an abundant growth of *Bacterium pneumosintes* in successive generations. A large inoculum, 0.2 to 0.5 cc. of fluid culture, should be used for seeding.

Preservation of Stock Cultures.

For the maintenance of our stock cultures we have heretofore relied upon the original ascitic fluid-tissue medium, in which *Bacterium pneumosintes* after a preliminary incubation of 5 to 7 days has remained viable at room temperature for 2½ years. Recently blood broth, without dextrose, has been used for the same purpose with encouraging results. The tests are incomplete and the limit of viability in this medium is not yet known, but *Bacterium pneumosintes* established in artificial culture appears to be a resistant organism, and probably requires transfer only at long intervals. We have also employed the method described by Swift[9] for the preservation of microorganisms by freezing and drying *in vacuo*. *Bacterium pneumosintes* withstands this process. The dried cultures are viable for at least 2 months.

In conclusion, we have at present available a number of cultural methods and culture media suitable for a variety of purposes.

For primary isolations from filtered nasopharyngeal secretions, or from the lung tissues of affected animals, the Smith-Noguchi ascitic fluid-rabbit kidney medium probably insures success in the largest percentage of cases. It was in this medium that the original

[9] Swift, H. F., *J. Exp. Med.*, 1921, xxxiii, 69.

isolations were made and the morphological and cultural characteristics of *Bacterium pneumosintes* were first observed.

For the maintenance of stock cultures also, the ascitic fluid-fresh tissue medium is preferred. Blood broth probably may be substituted in the case of well established cultures. *Bacterium pneumosintes* withstands freezing and drying *in vacuo* and probably is viable *for long periods in the dry state.*

For the demonstration of sparse growths of the microorganisms in the tissue medium, blood agar plates in an anaerobic jar have advantages over the microscope. They are useful also for the purification of contaminated cultures.

Finally, for immunization and for serological reactions requiring considerable quantities of bacteria with a minimal amount of foreign protein, dextrose broth cultures, enriched by the growth of another microorganism, furnish the most useful product. Suitable suspensions may be obtained also from blood agar plates or from growths in the dialysate liquid of collodion sacs containing the culture medium.

These methods of cultivation are being used in studies of the nasopharyngeal secretions of influenza patients obtained during the present recurrence of influenza in New York City. The results of these studies will be reported in subsequent papers of this series. ·

SUMMARY.

After artificial cultivation for a period of over 3 years *Bacterium pneumosintes* has maintained its original morphological and cultural characteristics, when grown in the original medium. Adaptation to a saprophytic existence has been accompanied by a loss of pathogenicity. Our strains now grow readily under strictly anaerobic conditions in a variety of media with peptone broth as a base, enriched with fresh tissue, blood, or by the growth of other bacteria. Surface colonies have been obtained on blood agar plates in an anaerobic jar. These various methods of cultivation are adapted to special purposes. In broth cultures *Bacterium pneumosintes* grows in larger forms than in the ascitic fluid-tissue medium, but the identity of the microorganisms is proved by their serological reactions and by reversion to the minute forms on transfer to the original medium.

EXPLANATION OF PLATE 67.

FIG. 1. *Bacterium pneumosintes*, Strain 16, in the twenty-fourth generation. Originally isolated March 30, 1919. Film from a collodion sac dialysate of ascitic fluid-rabbit kidney medium. × 1,000.

FIG. 2. *Bacterium pneumosintes*, same strain. Film from a culture in dextrose broth and rabbit kidney medium. × 1,000.

FIG. 3. *Bacterium pneumosintes*, same strain. Film from a collodion sac dialysate of ascitic fluid-rabbit kidney medium seeded from a culture in dextrose broth and rabbit kidney. Note the reversion to the original form. × 1,000.

MUTATION OF THE BACILLUS OF RABBIT SEPTICEMIA.

By PAUL H. De KRUIF, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 38.

INTRODUCTION.

Two varieties of microbe have been shown to exist in cultures of the rabbit septicemia bacillus (1). These organisms have been designated as Types D and G. Type D is the microbe invariably obtained at necropsy of rabbits dying from natural infection. Type G appears after artificial culture has been carried out for some time. It was important to determine whether the two varieties coexist in cultures isolated from infected rabbits, or whether Type G arises by mutation from Microbe D. Much of the work on mutation of bacteria has been criticized because of failure to employ pure-line strains, that is to say, cultures arising from single organisms. Since the primary isolations of Microbe D were made from colonies which conceivably might arise from two or more organisms, it would be unjustifiable to conclude that the original Type D had changed into the microbe of the G variety. Consequently, the experiments about to be reported were performed with pure-line strains isolated from a Type D culture by the Barber method (2).

Differential Characteristics of Microbes D and G.

These have been given in detail in a preceding communication (1) but will be briefly summarized here.

Type D is very virulent for rabbits, grows diffusely in liquid media, and yields smooth *fluorescent* colonies on serum agar. The acid agglutination optimum lies between pH 3.5 and pH 3.0 (Michaelis-Beniasch buffer series (3)). Type G is of very low virulence for rabbits, exhibits granular growth in fluid media, and grows in the form of slightly irregular, translucent, *non-fluorescing*

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colonies on serum agar. Its acid agglutination optimum lies between pH 5.0 and pH 3.5.

There is no noticeable morphological difference between the two varieties. Their fermentation reactions are indistinguishable. Immunization and agglutination absorption experiments indicate that they are antigenically homologous.

Method of Detection of Microbe G in Cultures in Which Microbe D Predominates.

This is made comparatively easy by the striking difference in appearance of the colonies on streaked serum agar plates, especially when the latter are held at certain angles before artificial light.

The tube of liquid medium supposed to contain the mixture of Types D and G is thoroughly shaken in order to bring into suspension clumps of Type G which may possibly have sedimented. A large
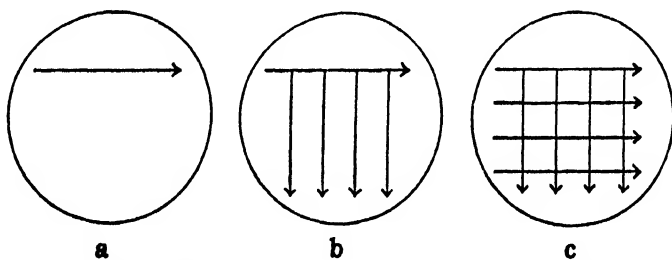
TEXT-FIG. 1, *a* to *c*. Diagrammatic sketch of the method of streaking serum agar plates.

platinum loop of the material is then streaked over the surface of 10 per cent rabbit serum agar in Petri dishes of 15 cm. diameter.

The loop is then sterilized, and successive streaks are made at right angles to the first one and starting from it. Then, without sterilizing the loop, it is passed across these second streaks and parallel to the original one. The resulting growth will therefore have a checkerboard appearance. The growth is heavy in the upper left-hand corner, the point where the loop was first applied. It will be much scantier in the lower right-hand corner. The successive stages of the operation are shown in the diagrammatic sketches in Text-fig. 1, *a* to *c*.

The result may be seen in Fig. 1. In this figure the Type D colonies can be readily detected, though in the minority. They are white while the Type G colonies appear gray.

The comparative scantiness of the colonies in the lower latitudinal streaks makes it possible to make rough quantitative estimates of the relative proportion of the Type D and G colonies.

Isolation of Pure-Line Strains.

The rabbit septicemia bacillus is rather exacting in its nutritive requirements, and it is necessary to make fairly generous seedings during routine transplant. As a result, difficulty was at first experienced in obtaining positive cultures by the Barber method. This was overcome by performing the entire operation in undiluted rabbit serum. The cultures used for fishing had been incubated in undiluted rabbit serum for 3 hours. Minute drops of these young cultures were placed in the usual manner in the Barber cell, examined by oil immersion lens, and when found to contain single organisms, fished to undiluted rabbit serum, and incubated. The percentage of positive cultures obtained by this method is relatively high. On the other hand, in plain or serum broth the results are very unsatisfactory.

EXPERIMENTAL.

The following observation led to the experiments about to be presented. Several strains of Microbe D, isolated at necropsy from rabbits dead of bronchopneumonia, were carefully purified as follows: A 6 hour serum broth culture was diluted in broth to 10^{-8} cc. of the original. Plates in serum agar made from this dilution usually showed from two to five colonies. One of these was fished to serum broth, and as soon as a cloud appeared (about 6 hours) the dilutions were again made and 10^{-8} cc. plated as before. This process was repeated four times in all. The young cultures used for these dilutions showed few or no clumps. The high dilutions—10^{-8}, 10^{-7}, and 10^{-6} cc.— when plated, yielded numbers of colonies that increased in direct proportion with the lowering of the dilution. That is to say, 10^{-8} plate, 2 colonies; 10^{-7} plate, 19 colonies; 10^{-6} plate, 205 colonies. These considerations make it certain that at some, and probably at each time during the fishing process a colony was obtained arising from *one* organism.

The Type D strains so purified were now subcultured daily in undiluted rabbit serum and in plain broth. Each culture was streaked

on 10 per cent rabbit serum agar in the manner just described. Careful inspection for Type G colonies was made. From the twentieth to the twenty-fifth passage, these began to make their appearance in small numbers in the plain broth series. None could be demonstrated in the plates made from undiluted serum.

One tube of the plain broth series which had shown *no* Type G colonies was allowed inadvertently to stand for 4 days at room temperature. It was then streaked, and large numbers of Type G forms appeared on the serum agar plate. None were present in a 4 day serum culture of the same Type D strain. The Type G colonies, subcultured from the plain broth plate, bred true to type and showed all of the characteristics previously described. It was concluded that an undoubted mutation of the original Type D strain had occurred. Eight pure-line strains were then obtained by the Barber method and the following experiments made, to determine, if possible, the conditions favoring and inhibiting the D → G transformation.

Experiment 1. D → G Mutation in Plain Broth, Serum Broth, and Undiluted Rabbit Serum.—0.05 cc. of a 6 hour undiluted serum culture of Microbe D, pure-line, was inoculated into three large test-tubes containing 10 cc. respectively of undiluted rabbit serum, 5 per cent rabbit serum broth, and plain broth. The tubes were placed in the incubator at 37°C., and streaked on serum agar plates by the method just described, at intervals covering a period of 109 hours. The plates were incubated for 24 hours and then inspected for the appearance of Type G colonies. The serum culture used for inoculation was streaked and observed to contain only Type D. The result of the experiment is given in Table I.

The result of Experiment 1 indicates that the ageing of a culture plain broth causes Microbe D to change into Type G. The addition of a small amount of serum does not greatly inhibit this change. On the other hand, undiluted rabbit serum has a very marked inhibitory effect.

All of the pure-line strains under study have been found to undergo this mutation when allowed to age in plain broth, but do so with varying rapidity and completeness. The Type G colonies, fished into plain broth or undiluted serum, remained true to type indefinitely. They showed no tendency to revert to the parent D form even when transplanted continuously in undiluted rabbit serum, a medium which is antagonistic to the original change.

It was thought that it might be possible to demonstrate mutation-enhancing properties in plain broth cultures of Microbe D. The following experiment examines this question.

Experiment 2. Effect of Type D Culture Filtrates on the Mutation Rate of Microbe D.—A pure-line strain of Microbe D was planted into a series of flasks containing plain broth (pH 7.4). These were incubated at 37°C. After 6, 24, 48, and 72 hours, a flask was removed, centrifuged, and filtered through small Berkefeld candles   The filtrates upon test proved to be sterile. A slight change in reaction had occurred, the 6 and 24 hour filtrates giving pH 7.1, the 48 hour pH 7.0, and the 72 hour pH 6.9. The filtrates were now divided into two

TABLE I.

D → G Mutation in Plain Broth, Serum Broth, and Undiluted Rabbit Serum.

Strain used for inoculation.	Medium.	Proportion of Types D and G.							
		9 hrs.	24 hrs.	36 hrs.	48 hrs.	60 hrs.	72 hrs.	85 hrs.	109 hrs
0.05 cc. of 6 hr. serum culture; pure-line strain; Type D.	Undiluted rabbit se-rum.	D	D	D	D	D	D	D	D
	5 per cent serum broth.	"	"	"	"	"	" +2G.	" + sev-eral G.	" + G (about 70:30).
	Plain broth.	"	"	"	" + few G.	" + few G.	D + mod-erate number of G.	D + many G.	D + G (about 50:50).

parts, half was titrated back to pH 7.4 with 0.1 N NaOH, the remainder was left without treatment. 10 cc. of each filtrate were placed in large test-tubes. These tubes were now seeded with 0.05 cc. of serum culture of pure-line strain, Type D. Controls, consisting of similar amounts of sterile broth incubated for 6 and for 72 hours, and of undiluted rabbit serum, were similarly seeded. All of the tubes were now incubated at 37°C., and streaks made on serum agar plates at intervals up to 7 days, 10 hours. Growth occurred in all of the filtrates, although a considerable lag period was evident in those of 48 and 72 hours. The results of the experiment are presented in Table II.

TABLE II.

Effect of Type D Filtrates on the Mutation Rate of Microbe D.

Tube No.	Medium.	pH	Proportion of Types D and G.									
			12 hrs.	28 hrs.	43 hrs.	60 hrs.	72 hrs.	84 hrs.	106 hrs.	120 hrs.	138 hrs.	176 hrs.
1	6 hr. filtrate.	7.1	D	D	D	D	D	D +1 G.	D	D	D	D
2	6 " "	7.4	"	"	"	"	"	D	"	D + 2 G.	D + few G.	"
3	24 " "	7.1	"	"	"	"	" +2 G	" +1 G.	"	" + few G.	D	D + few G.
4	24 " "	7.4	"	"	"	"	D	D	"	" + " "	"	D
5	48 " "	7.0	"	"	"	"	"	"	D +1 G.	D + moderate number of G.	D + few G.	D + few G.
6	48 " "	7.4	"	"	"	"	"	"	D	D + moderate number of G.	" + " "	" + " "
7	72 " "	6.9	"	"	"	"	"	" +1 G.	D + moderate number of G.	D + G (50:50).	D + G (50:50).	D + G (50:50).
8	72 " "	7.4	"	"	"	"	"	D	D + moderate number of G.	D + G (50:50).	D + G (40:60).	D + G (50:50).
9	Sterile broth (37°C. 6 hrs.).	7.4	"	"	"	"	"	" +12 G.	D + many G.	D + G (40:60).	D + G (40:60).	D + G (40:60).
10	Sterile broth (37°C. 72 hrs.).	7.4	"	"	"	"	"	D +6 G.	" + "	D + G (30:70).	D + G (30:70).	D + G (50:50).
11	Undiluted serum.	7.4	"	"	"	"	"	D	D	D	D	D

A glance at Table II shows that the expectation of the discovery of conditions enhancing mutation was not verified. In fact, the opposite property is seen to be present in the 6, 24, and 48 hour filtrates. Contrary to expectation, the number of Type G colonies arising in the 6 and 24 hour filtrates was extremely small, and comparatively few appeared in that of 48 hours. In the 72 hour filtrate the mutation rate paralleled that of the control broth. The mutation had reached 50 per cent or more in 176 hours. In undiluted rabbit serum no Type G colonies appeared at any time during the experiment. Early filtrates from Type D cultures exhibit a markedly antagonistic action to the mutation. Little or no difference is to be observed between the 72 hour filtrate of reaction pH 6.9 and its companion tube, which had been titrated back to pH 7.4. It would appear that the slight change in reaction has no effect upon the mutation. Many of the Type G colonies were subjected to the differential tests, and proved in all cases to be authentic Type G cultures. They did not revert to Type D.

An effort was now made to discover, if possible, the constituents of plain broth that encourage the D → G mutation. Plain beef infusion was the first of these elements subjected to test. The beef infusion was prepared in exactly the same way as in the preparation of plain broth. It was titrated to pH 7.4. The only difference between it and the plain broth ordinarily used was the absence of peptone (Fairchild's) and of Na_2HPO_4, which is added for buffer purposes.

Experiment 3. D → G Mutation in Beef Infusion and Other Media.—0.05 cc. of a serum culture of a pure-line strain, Type D, was seeded into large test-tubes containing respectively 10 cc. of 6 hour Type D filtrate, of plain broth, of 5 per cent rabbit serum broth, of beef infusion, of 5 per cent rabbit serum-beef infusion, and of undiluted rabbit serum. The material used for seeding was streaked on serum agar plates and proved to contain only Type D microbes. The plain broth was made up from the beef infusion used in the experiment. All of the tubes were placed at 37°C. in the incubator and tested by streaking on serum agar plates at intervals up to 18 days. The results are given in Table III.

Table III indicates that beef infusion is very unsuitable to the D → G change. In 228 hours, frequent streaking of 10 per cent serum agar plates revealed only one Type G colony in this medium. In 18 days

a considerable mutation had taken place. The process, therefore, can take place in this medium, but is undoubtedly greatly retarded in comparison with the mutation occurring in plain broth. It is interesting to note that in the beef infusion-serum mixture, onlyone Type G

TABLE III.

D → G Mutation in Beef Infusion and Other Media.

Period of incubation at 37°C.	Proportion of Types D and G.					
	Tube No.					
	1	2	3	4	5	6
	6 hr. Type D filtrate.	Plain broth.	5 per cent serum broth.	Beef infusion.	5 per cent serum-beef infusion.	Undiluted serum.
hrs.						
12	D	D	D	D	D	D
24	"	"	"	"	"	"
36	"	"	"	"	"	"
52	"	D + few G.	"	"	"	"
84	"	" + " "	"	"	"	'"
96	D + 3 G.	D + moderate number of G.	D + 1 G.	"	"	"
120	" + few G.	D + many G.	D	"	"	"
144	" + few G.	D + G (70:30).	"	D + 1 G.	"	"
173	" + few G.	D + G (30:70).	D + many G.	D	"	"
197	D	D + G (30:70).	D + many G.	"	D + 1 G.	"
228	"	D + G (40:60).	D	"	D	"
days						
18	"	D + moderate number of G.	"	D + G (95:5).	"	D + few G.

colony was observed over a period of 18 days. The 6 hour Type D filtrate showed its usual inhibitory activity. This was the same filtrate as that used in Experiment 2. It had been kept in the ice box for 9 days after that experiment. In undiluted rabbit serum no Type G colonies were observed for 228 hours. A few were revealed by the examination after 18 days. This indicates, again, that the process

D → G takes place in a variety of media, but that the tendency is far more marked in some than in others.

A further interesting fact is to be observed in Table III. It will be noted that in Tube 2 (plain broth) the proportional number of Type G colonies reaches a maximum after 173 to 197 hours, is less in 228 hours, and far less after 18 days. This is also true of the serum broth (Tube 3). This phenomenon has been observed many times and its analysis is demanded. The great decrease in the relative number of Type G colonies must be due to one of two causes. Either the mutant G forms die off more rapidly than do the parent D microbes, or an equilibrium is established in the tubes which results in a tendency of the newly mutated Type G to revert to the original Type D. Such reversion has never been noted in subcultures made by fishing Type G colonies. On the other hand, it is conceivable that an equilibrium might be set up in the original reaction tube.

TABLE IV.

D → G Mutation in 2 Per Cent Peptone (Fairchild's).

Period of incubation at 37°C., hrs.	15	30	48	72	96	134	146	183
Proportion of Types D and G.	D	D + 3 G.	D	D	D + G (40:60).	D + few G.	D	D

The striking retardation of D → G mutation in beef infusion led to the examination of peptone as a possible factor in the hastening of the process. Preliminary experiments indicated that it was necessary in order to obtain growth to seed relatively large amounts of Microbe D into the peptone solutions.

Experiment 4. D → G Mutation in 2 Per Cent Peptone (Fairchild's).—The peptone solution was made up in distilled water and titrated to reaction pH 7.4. A large test-tube containing 10 cc. of this medium was then seeded with 0.5 cc. of a 10 hour culture of pure-line strain, Type D, in peptone. The tube was incubated at 37°C. for 183 hours and tests on serum agar plates were made at intervals during this time. The result is given in Table IV.

Marked mutation is seen to occur in peptone solution, and the phenomenon of a decrease in the number of Type G colonies after a maximum is strikingly manifest in this experiment.

TABLE V.

$D \rightarrow G$ Mutation in Various Concentrations of Peptone (Fairchild's)

Tube No.	Peptone 20 per cent.	Beef infusion.	Final per cent of peptone.	24 hrs.	48 hrs.	72 hrs	96 hrs.	120 hrs.	144 hrs.	192 hrs.
	cc.	cc.								
1	10.0		20.0	D	D + 7 G.	D + 10 G.	D + G (50:50).	G + few D.	G + 2 D.	
2	5.0	5.0	10.0	D + 3 G.	D	D + many G.	D + G (90:10).	D + G (10:90).	D + G (10:90).	D + G (50:50).
3	2.5	7.5	5.0	D	D + G (90:10).	D + G (40:60).	D + G (15:85).	D + G (2:98).	D + G (2:98).	D + G (50:50).
4	0.5	9.5	1.0	"	D	D	D	D + few G.	D + few G.	D
5	0.1	9.9	0.2	"	D + 3 G.	"	"	" + " "	D	"
6	Plain broth	10.0		"	D	"	"	" + 2 G.	"	"
7	Rabbit serum un-diluted.			"	D + few G.	D + 8 G.	"	D + many G.	D + many G.	D + few G.
8				"		D	D + 1 G.	D	D	D

The next experiment dealt with the effect of various concentrations of peptone on the process. A stock solution of 20 per cent Fairchild's peptone was prepared and titrated to pH 7.4.

Experiment 5. D → G Mutation in Various Concentrations of Peptone (Fairchild's).—Eight large tubes were prepared containing respectively 20 per cent peptone solution undiluted, 20 per cent peptone solution diluted to varying degrees with beef infusion, beef infusion alone, plain broth, and undiluted rabbit serum. The pH of all of these media was 7.4. They were seeded with 0.1 cc. of a pure-line strain of Microbe D, 6 hour culture in rabbit serum, which showed only Microbe D when streaked on serum agar. The tubes were then placed in the incubator at 37°C. and streaked on serum agar plates at intervals up to 8 days. The results of the experiment are given in Table V.

Table V shows in a very striking manner the marked effect of high concentration of peptone on the mutation. The process occurs much more rapidly than is usually the case in plain broth, and, what is more, in the case of 20, 10, and 5 per cent peptone, goes much nearer to completion. That is to say, the mutation instead of involving 30 to 40 per cent of the organisms, reaches 90 per cent and in some cases arrives almost at completion. On the other hand, the tubes with very low concentrations of peptone or with beef infusion alone and with undiluted rabbit serum show a very few Type G colonies during the entire period of the experiment and in the last two tests proved to contain nothing but Type D. The decrease in relative number of Type G colonies is again strikingly apparent in Tubes 2 and 3, 192 hour test.

It cannot be said that the presence of the peptone causes the mutation D → G, since the change occurs occasionally, though only to a small extent, in beef infusion and in undiluted rabbit serum. But it is certain that the presence of peptone in suitable concentrations greatly accelerates a reaction toward which a tendency already exists. It is interesting that four pure-line strains, kept on ice in undiluted rabbit serum for 3 months without passage, showed no evidence of the appearance of Type G colonies when subsequently transplanted.

Some preliminary experiments have been made in regard to the effect of varying C_H+ of the medium upon the D → G process. These of course are limited by the range within which good growth occurs; *i.e.*, about pH 8.5 to pH 6.0. The results would indicate that

reaction of pH 6.0 distinctly retards the process, while it is, if anything, accelerated in reaction pH 8.5.

DISCUSSION.

Great confusion exists among bacteriologists in regard to the meaning of the term mutation. Its use in application to the experiments just described is considered to be entirely justified in the light of the definition of Dobell (4), who speaks of it as "a permanent change—however small it may be—which takes place in a bacterium and is then transmitted to subsequent generations." The Type G colonies arising in these experiments, and subcultured to undiluted rabbit serum, were frequently tested for the characters that distinguish them from the parent Type D. The mutant G forms were found in every case to possess little or no virulence, to grow in a granular fashion in fluid media, and to possess an acid agglutination point distinctly different from that of Type D. All of these characters persist throughout many passages in undiluted serum, a medium in which the original change seldom, if ever, takes place. It would seem that if a so called atavistic reversion were to take place, it would certainly occur in undiluted serum. This has never been observed.

The majority of authentic examples of bacterial mutation has been concerned with gain or loss in fermentative power. We refer here only to work performed with pure-line strains. Among these researches, the investigations of Benecke (5) and Kowalenko (6) deserve to be mentioned. These investigators found that Massini's (7) *Bacterium coli mutabile* gained the power of splitting lactose when cultivated in this sugar and that this power persisted indefinitely. The work of Müller (8) in regard to *Bacillus typhosus* is of a similar nature as is that of Burri (9) with *Bacterium imperfectum*. It would seem that the D → G process just described involves a much more fundamental change in the bacterial cell. The distinct difference in acid agglutination optimum is a remarkably regular occurrence. It is in the nature of a physical constant for each type and would imply an important change in the protoplasm of the organism. It is certain that this phenomenon is not confined to the hemorrhagic septicemia group, of which the rabbit septicemia bacillus is a member. Varieties showing granular growth character have been discovered in

typical cultures of paratyphoid organisms by Krumwiede (10), of *Bacillus dysenteriæ* Shiga by Arkwright (11) and Zoeller (12), and in cultures of *Bacillus coli* by Gratia (13). The way in which these have arisen has not been taken up by these authors, but it is quite certain to be found of a nature closely akin to the process just described.

SUMMARY AND CONCLUSIONS.

Type G microbes, discovered in pure cultures of the rabbit septicemia bacillus, have been demonstrated to arise from the parent D form by mutation.

The D → G mutation takes place in broth cultures of pure-line strains of Microbe D, when these are kept for several days without transplant at 37°C., or at room temperature, or in the ice box.

The mutation is greatly inhibited by filtrates from 6 and 24 hour cultures of Microbe D, and to some extent by filtrates from 48 hour cultures.

The process of transformation takes place to a very slight extent or not at all in undiluted rabbit serum, but Type G colonies subcultured to this medium do not revert to the parent D form.

The D → G change is strongly inhibited in cultures made in simple beef infusion, or in 5 per cent rabbit serum-beef infusion.

Peptone would seem to be the constituent of plain broth which favors the process. In high concentrations of peptone, the mutation is rapid and may reach a degree of 90 per cent of the total organisms in 5 to 6 days.

A distinct maximum of the relative number of Type G colonies as compared to the parent Type D is observable in plain broth and in some concentrations of peptone, when these are kept at 37°C. for some days without transplant. Subsequent tests show the concentration of Type G microbes to diminish.

The change in acid agglutination optimum exhibited by the mutant G forms implies a distinct change in bacterial protoplasm and would seem to be one of the most fundamental mutations so far described.

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EXPLANATION OF PLATE 38.

Fig. 1. 10 per cent rabbit serum agar plate showing mixed culture of Microbes D and G. Type G preponderates and has a grayish appearance. The Type D colonies are white. The arrows indicate type D colonies.

Fig. 1.

METHODS FOR THE ISOLATION OF FILTER-PASSING ANAEROBIC ORGANISMS FROM HUMAN NASO-PHARYNGEAL SECRETIONS.

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In November, 1918, during the pandemic of influenza, we isolated from the filtered nasopharyngeal secretions of influenza patients, and from the lung tissues of rabbits intratracheally inoculated with these secretions, a hitherto undescribed organism which we called *Bacterium pneumosintes.*[1] The same organism was recovered from similar sources during the recurrent wave of epidemic influenza in the spring of 1920. Within the last few weeks we have again isolated identical or similar organisms from the nasopharyngeal secretions of influenza patients. At present the identification of the recently recovered bacteria is not complete. But during our observation of the strains isolated in 1918–1919 and in 1920, we have noted certain unreported facts relating to the cultural and morphologic characters of *Bacterium pneumosintes* which have been useful in the isolation of the recently recovered strains. It is our purpose in this communication to report these facts briefly, in the hope that this information may be of use to others in the bacteriologic study of respiratory infections of obscure etiology.

Bacterium pneumosintes, as originally isolated, was a minute bacilloid body of regular form, with a length about two to three times its breadth, measuring from 0.15 to 0.3 micron in the long axis. It passed V and N Berkefeld filters, multiplied slowly only under strictly anaerobic conditions in a medium composed of human ascitic fluid and a fragment of fresh rabbit kidney, and withstood glycerolation for a period of months. The organism decolorized by Gram's method, and stained with the usual basic dyes, of which Loeffler's alkaline

1. Olitsky, P. K., and Gates, F. L.: Studies of the Nasopharyngeal Secretions from Influenza Patients, J. A. M. A. 76: 640 (March 5) 1921; J. Exper. Med, 33: 125 (Feb.) 1921; ibid. 33: 713 (June) 1921.

methylene blue was the most suitable. *Bacterium pneumosintes* was identified as the cause of the characteristic clinical and pathologic effects induced in rabbits injected intratracheally with the naso-pharyngeal secretions of influenza patients.

During artificial cultivation over a period of from two to three years, our strains of *Bacterium pneumosintes* have maintained their strictly anaerobic character. Hence it is necessary to cultivate them in an anaerobic jar, or under a petrolatum seal in mediums containing active reducing substances, such as fresh animal or vegetable tissue, dextrose or peptone. The stock cultures are being maintained in the medium originally used, human ascitic fluid and fresh rabbit kidney, under a petrolatum seal. In this medium the strains have retained their original cultural and morphologic characters, and require transfer only at long intervals.

The ascitic fluid and rabbit kidney medium is an expensive and difficult medium to prepare, and we have tried various simpler methods of cultivation, some of which have been successful. The basis of the mediums which have proved to be suitable is a 1 per cent dextrose, 1 per cent peptone, 0.5 per cent sodium chlorid, beef infusion broth, titrated to a hydrogen ion concentration of 7.4 on the .Sörensen scale. This broth must be enriched with fresh rabbit blood, rabbit kidney tissue, fresh vegetable tissue[2] or by the growth of other organisms, as will be described. Solid and semisolid mediums may be made by the addition of agar in the proper proportion.

In fluid mediums in an anaerobic jar, or under a petrolatum seal, *Bacterium pneumosintes* grows in a diffuse cloud, too finely divided to give a typical bacterial shimmer. In a previously incubated medium, in which anaerobic conditions are already established, the cloud may become visible in from sixteen to twenty-four hours. The limit of growth is reached on the third to the seventh day at a hydrogen ion concentration of from 5.2 to 5.3. The cloud remains in suspension several days longer, and gradually falls to the bottom of the tube as a smooth, cream-colored sediment.

Surface colonies have been obtained on 5 per cent rabbit blood agar plates incubated in an anaerobic jar. Dextrose is not necessary

2. Avery, O. T., and Morgan, H. J.: Proc. Soc. Exper. Biol. & Med. 19: 113 (Dec.) 1921.

under these conditions. The jar must be capable of establishing strictly anaerobic conditions so that an indicator tube of methylene blue in dextrose broth is decolorized over night. We recommend the jar described by Brown,[3] which depends on the union of the oxygen with hydrogen under the catalytic influence of palladinized asbestos heated by a small resistance coil in a wire-screened chamber.

Grown under anaerobic conditions on the surface of blood agar plates, *Bacterium pneumosintes* forms extremely minute, round, convex, amorphous colonies with an entire edge and a colorless or grayish translucence. The medium is not precipitated or decolorized. The colonies are usually discrete, on account of their minute size, but may coalesce in the most crowded areas. The rather smeary growth forms a smooth suspension.

In stained smears, *Bacterium pneumosintes*, grown in enriched dextrose broth or on agar plates, develops as a minute, spindle-shaped bacillus, somewhat longer than the forms already described. The length may be increased to 1 micron, making the bacteria obviously bacillary. Short chains and diploforms are found. On old blood agar plates, involution forms have been observed. The longer, rod-shaped organisms have been fully identified with the original minute forms by serologic tests, and revert to the minute forms on transfer to the original ascitic fluid-rabbit kidney medium.

Rabbits immunized with *Bacterium pneumosintes* develop specific precipitins, agglutinins, bacteriotropins and complement-fixing bodies.

The growth of *Bacterium pneumosintes* in cultures contaminated with pneumococci, staphylococci, streptococci and hemophilic bacilli has already been reported.[1] Recently we have studied this symbiotic relationship, and find that other organisms may be used as a method of enrichment of the culture medium.

The most suitable organisms that we have tested for this purpose are *B. mesentericus*, *Bacillus coli*, *Bacillus typhosus*, and the pneumococcus Type III. In the case of *B. mesentericus*, the organism is grown for from eight to sixteen hours in aerated dextrose broth. A seal of petrolatum then checks growth promptly, since *B. mesentericus* is a strict aerobe, and an anaerobic condition is quickly established in the medium. If *Bacterium pneumosintes* is now inoculated, it

3. Brown, J. H.: J. Exper. Med. 33: 677 (June) 1921.

grows rapidly and kills vegetative forms of *B. mesentericus* by acid production. Unless spores were introduced when the broth was seeded, a pure culture of *Bacterium pneumosintes* may often be obtained on the fourth or fifth day. It is necessary to prepare each lot of mediums with fresh transplants of *Bacillus mesentericus*. The few viable organisms, or spores, that may be carried over are insufficient to establish a growth in the second generation under a petrolatum seal.

Bacillus coli, *Bacillus typhosus* or pneumococcus Type III is grown in dextrose broth under a petrolatum seal until the bacterial cloud just becomes visible. Then the organisms are killed by exposure to 100°C. for from fifteen to thirty minutes. In the case of all these mediums, time is saved by a primary incubation to establish anaerobic conditions and insure sterility.

On the basis of these observations, we offer the following suggestions for the bacteriologic examination of nasopharyngeal secretions in cases of respiratory disease which apparently are not due to ordinary aerobic bacteria:

Nasopharyngeal washings should be obtained by a careful syringing of the nose and pharynx with from 20 to 40 c.c. of warm sterile salt solution, the washings being collected in a sterile kidney basin and transferred to a bottle with glass beads in which they may be thoroughly emulsified by shaking. After samples of the whole nasal washings have been spread on plates for aerobic cultivation, the remainder should be filtered through a tested candle, preferably a Berkefeld V. The filtered nasal washings should be inoculated in fairly large amounts, from 1 to 2 c.c., into human ascitic fluid-rabbit kidney medium and into incubated blood broth under a petrolatum seal. Broth enriched by the growth of other organisms, or with fresh vegetable tissue, may also be tried. Blood agar plates should be spread with the filtrate and incubated at least seven days in an anaerobic jar. The use of aerobic and anaerobic controls for each method of cultivation is essential.

At the same time, healthy rabbits should be intratracheally inoculated with 3 cc. of the whole or filtered washings. Reference must be made to our former papers[1] on the subject for the details of this procedure and the effects which characterize the presence of an

active, living agent in the inoculated material. In several instances we have obtained cultures after passage through the rabbits' lungs which were missed on cultivation of the original filtrate.

The use of blood plates in an anaerobic jar has proved especially useful for primary isolations from the filtered nasopharyngeal secretions and for the demonstration of organisms in a second generation after transfer from an original ascitic fluid-kidney medium. This medium seems to assure growth in the largest percentage of cases, but the ocular demonstration of the sparse primary growths is often exceedingly difficult and unconvincing because of the minute size of filter-passing organisms and the presence of protein precipitate from the medium. Colony formation on blood plates, on transfer from the tissue medium, has therefore been of great assistance to us.

It seems not unlikely that *Bacterium pneumosintes* is only one of a number of related or unrelated, anaerobic, filter-passing organisms which may be obtained by appropriate methods from human sources. An endeavor should be made to obtain as many examples as possible of this new group or class of micro-organisms. Their classification and etiologic significance may await subsequent investigation.

VIRULENCE AND MUTATION OF THE BACILLUS OF RABBIT SEPTICEMIA.

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INTRODUCTION.

Types D and G, bacillus of rabbit septicemia, have been shown to possess greatly differing degrees of virulence (1). Type G, of low virulence, has been demonstrated to arise as a mutant from the primordial highly virulent Type D (2). It is the purpose of this paper to inquire into the fixity of the character of virulence of the two types and to determine the relation that mutation bears to this function of invasibility.

Method.

The method of virulence determination employed in the following experiments is identical with that described in a previous paper (1). Briefly, it consists in the injection of varying amounts of 6 hour 10 per cent rabbit serum broth cultures, intrapleurally, into young rabbits of about 600 gm. weight. The dilutions of the test culture are carried out in plain broth, pH 7.4, and are increased by 10's. The dilutions are designated as follows: 0.0001 cc. of the original culture is written 10^{-4} cc., which of course is an abbreviation for 1×10^{-4}. The injections were invariably made immediately after dilution. The highest dilution was injected first, this procedure permitting the same syringe to be used for all injections. The final volume of each inoculation was in all cases 0.5 cc.

EXPERIMENTAL.

The persistence of the character of virulence of Type D is demonstrated by the following observations.

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Experiment 1. Persistence of the Virulence of Strain R19, Type D.—Strain R19, Type D, was isolated on October 20, 1920, from the heart's blood of a rabbit dead of bronchopneumonia. The necropsy showed the typical bronchopneumonia and fibrinopurulent pleuritis and pericarditis, caused by the rabbit septicemia bacillus. This culture was placed in 10 per cent rabbit serum agar and transplanted at intervals of 7 days—incubation at 37°C. for 8 hours, the remainder of the time in the ice box. Virulence was tested on November 5, 10, and 14, 1920, and February 2, 1921; that is, 16, 21, 25, and 105 days after isolation. The results are summarized in Table I.

TABLE I.

· *Persistence of the Virulence of Strain R19, Type D.*

Strain.	Length of time after isolation.	Weight of rabbit.	Age of serum broth culture injected.	Amount injected intrapleurally.	Result.
	days	*gm.*	*hrs.*	*cc.*	
R19-D	16	550	6	10^{-4}	Died in 15 hrs.*
		550		10^{-3}	" " 13 "
		560		10^{-1}	" " 13 "
R19-D	21	600	6	10^{-4}	" " 14 "
		650		10^{-5}	" " 14 "
R19-D	25	525	6	10^{-9}	Survived.
		515		10^{-8}	Died in 12 hrs.
R19-D	105	650	6	10^{-8}	" " 15 "
·		650		10^{-7}	" " 15 "
		650		10^{-4}	" " 15 "
		650		10^{-5}	" " 15 "

* The necropsy showed typical bronchopneumonia, fibrinopurulent pleuritis, and pericarditis.

Table I indicates that the virulence of this strain remains at a very high level over a comparatively long period of time. The high dilutions (10^{-8}, 10^{-7}) were plated in serum agar. 10^{-8} cc. yielded from three to eight colonies, showing the number of viable organisms in a 6 hour culture to vary from 300 to 800 million per cc. ·Strain R19-D was the most violently invasive of any of the cultures isolated. It is interesting to observe that the time of death of the test animals shows very little variation (12 to 15 hours). For the majority of virulent

Type D strains this is somewhat longer; *i.e.*, from 30 to 48 or 72 hours. Other Type D strains have been subjected to similar tests and, like Strain R19-D, exhibit a like constancy in maintenance of a high degree of virulence.

It was of interest to determine whether or not individual members of a single Type D culture were possessed of greatly varying degrees of virulence.

Experiment 2. Virulence of Six Pure-Line Strains, Type D, Fished from a Single Culture.—Six pure-line strains of Type D, R15, were fished by Barber pipette (3) from a 3 hour undiluted rabbit serum culture. These were seeded into undiluted rabbit serum and after several passages in this medium were transplanted to 10 per cent rabbit serum broth, incubated for 6 hours at 37°C., and tested for virulence by the usual technique. The results are summarized in Table II.

TABLE II.

Virulence of Six Pure-Line Strains, Type D, Fished from a Single Culture.

Strain	Weight of rabbit.	Age of serum broth culture injected	Amount injected intrapleurally.	Result.
	gm.	*hrs.*	*cc.*	
B-D$_1$	600	6	10^{-6}	Died in 36 hrs.*
	580		10^{-4}	" " 36 "
B-D$_2$	600	6	10^{-6}	" " 36 "
	600		10^{-4}	Survived.
B-D$_3$	610	6	10^{-6}	Died in 48 hrs.
	590		10^{-4}	" " 47 "
B-D$_4$	600	6	10^{-6}	" " 36 "
	600		10^{-4}	" " 36 "
B-D$_5$	610	6	10^{-6}	" " 36 "
	610		10^{-4}	" " 48 "
B-D$_6$	600	6	10^{-6}	" " 36 "
	590		10^{-4}	" " 36 "

* The necropsies were typical in all cases.

Table II indicates that there is very little variation in different individuals of a Type D culture. All of the six serum broth subcul-

tures from the pure-line strains were streaked on serum agar at the time of the virulence test and were found to contain Type D organisms only.

It is well known that various microorganisms attenuate quickly when they are isolated from the animal body and grow upon artificial media. This loss of virulence appears especially rapidly when the media employed are not enriched with blood, serum, or ascitic fluid. Wadsworth and Kirkbride (4) found that the virulence of pneumococci could be maintained at a high level for a long period when the culture was transplanted continuously at 8 hour intervals in plain broth, but that it quickly decreased when 24 hours were allowed to elapse between passages. Various observations having indicated that the virulence of Type D shows little variation, an experiment was planned to determine whether decidedly unfavorable conditions would depress this function. It has been shown in a previous communication that pure-line strains of Microbe D undergo mutation into Type G when allowed to stand without transplant for some days in plain broth. This process does not, however, go to completion under these conditions. A certain proportion of Type D remains as is evidenced by streaking such cultures on serum agar plates. It was proposed, therefore, to test the virulence of those Type D organisms which remain without change and at the same time to find out whether the newly mutated Type G colonies would yield cultures of characteristically low invasive power.

Experiment 3. Persistence of the Virulence of Type D in Broth Cultures Kept for 9½ Days at 37°C. without Transplant; Lack of Virulence of Type G Mutants Arising in This Culture.—0.05 cc. of an 18 hour rabbit serum culture, Strain A-D, R15, was seeded into a large test-tube containing 10 cc. of plain broth, pH 7.4. The tube was capped with tin-foil and placed in the incubator at 37°. The tube was removed from the incubator at 24 hour intervals and a loop of its contents streaked on 10 per cent rabbit serum agar plates by the method described in a preceding paper (2). Type G colonies began to appear on the plates of the 52 hour test, and at 228 hours (9½ days) Type G showed preponderance over Type D, the ratio between D and G being 40:60. Characteristic fluorescent Type D and non-fluorescent Type G colonies were now carefully fished to 10 per cent rabbit serum broth. Good growth had occurred in broth of the subcultures at 8 hours, the appearance of Type D being diffusely turbid, that of Type G very granular. Both tubes were now carefully shaken up, diluted to 10^{-7} cc. of the

original in plain broth, pH 7.4, and immediately injected intrapleurally into 700 gm. rabbits. The results of this experiment are recorded in Table III.

TABLE III.

Persistence of the Virulence of Type D in Broth Cultures Kept for 9½ Days at 37°C. without Transplant; Lack of Virulence of Type G Mutants Arising in This Culture.

Type.	Age of serum broth culture injected.	Weight of rabbit.	Amount injected intrapleurally.	Result.
	hrs.	gm.	cc.	
Type D; from plain broth culture; 228 hrs.; 37°C.	8	700	10^{-7}	Died in 42 hrs.*
		710	10^{-6}	" " 36 " *
		700	10^{-5}	" " 36 " *
		690	10^{-4}	" " 36 " *
		700	10^{-3}	" " 42 " *
		720	10^{-2}	" " 22 " *
		680	10^{-1}	" " 18 " *
Type G; from plain broth culture; 228 hrs.; 37°C.	8	700	10^{-4}	Survived.
		710	10^{-3}	"
		700	10^{-2}	"
		710	10^{-1}	"
		700	5×10^{-1}	Died in 15 days. †

* Necropsy typical.

† Necropsy showed no evidence of infection; heart's blood sterile.

It will be observed from Table III that the subculture from the Type D colony was very virulent. Injected over a range of dilutions from 10^{-7} to 10^{-1} cc., it proved fatal in every case. On the other hand, 0.1 cc. of the subculture from the Type G colony failed to produce an appreciable ill effect. The rabbit receiving 0.5 cc. succumbed in 15 days, but necropsy failed to reveal the characteristic bronchopneumonia and fibrinopurulent pleuritis and pericarditis, and the heart's blood culture proved sterile. This experiment is a clear demonstration that Microbe D is able to preserve its virulence under conditions generally considered to be unfavorable, while the mutant Type G forms that have arisen in the same culture show the lack of virulence that characterizes this type. It is apparent from the foregoing experiment that the virulence of a culture containing both Types D and G is proportional to the number of the D variety remaining in the culture.

If this be true, it is conceivable that the attenuation of a Type D culture in plain broth may be due to a D → G mutation and gradual increase in the number of Type G individuals. This augmentation might take place either by a constantly increasing tendency of the Type D forms to mutate, or by an actual overgrowth of Type D by the mutant G variety. If Type G should come finally to supplant the parent Type D completely, the formerly virulent culture should be almost completely attenuated. This idea was put to test in Experiment 4.

Experiment 4. Relation of the Virulence of a Plain Broth Culture to the Proportions of Types D and G Present.—Microbe D,R15, was transplanted daily for 25 days in plain broth, pH 7.4. From time to time the culture was streaked on 10 per cent serum agar plates. No Type G forms were observed. At the twenty-fifth passage the virulence of the culture was tested by the usual technique. At the thirtieth plain broth passage a few Type G colonies were detected on the serum agar plate. The culture was now transplanted every 48 hours. The relative number of Type G colonies increased rapidly. At the 51st passage Type G was found to preponderate greatly over Type D. The virulence of the whole culture was again tested at this point. The period of time between transplants was lengthened to 5 days. At the 56th passage serum agar plates revealed nothing but Type G. Microbe D had completely disappeared. The virulence was again tested. The results of the experiment are summarized in Table IV.

The results of Experiment 4, as seen in Table IV, can be summed up briefly as follows: At the twenty-fifth passage in plain broth no Type G colonies had arisen and the virulence was approximately equal to that of the original serum broth culture of Type D, 10^{-6} cc. proving fatal to 600 gm. rabbits. At the 51st passage, 2 months later, there was a large preponderance of Type G. The virulence had fallen to 10^{-4} cc. At the 56th passage, 5 days later, no Type D colonies could be demonstrated. Either complete D → G mutation had occurred, or the originally mutated Type G had completely outgrown Type D. 0.1 cc. of the culture failed to produce a fatal effect. The attenuation of this culture is to be referred to the gradual replacement of the primordial Type D by the mutant G form. It is possible to predict the virulence of a culture from the proportion of the two types present, as evidenced by the serum agar plate streak method. It is possible to procure subcultures of very high or very low virulence by selection of one type or the other, so long as any of Type D remain.

While the virulence of Type G, isolated from Type D cultures, is very low, large amounts of undiluted culture may occasionally cause fatal infections, especially in young rabbits. Adult rabbits of 1,200 to 1,800 gm. weight have not been observed to succumb, even when

TABLE IV.

Relation of the Virulence of a Plain Broth Culture to the Proportions of Types D and G Present.

No of passages.	Interval between transplants.	Proportion of Types D and G	Amount injected intrapleurally.*	Result.	Necropsy.
	days		*cc*		
25	1	D only.	10^{-6}	Died in 36 hrs.	Typical. Type D in heart's blood.
			10^{-5}	" " 24 "	Typical. Type D in heart's blood.
			10^{-4}	Survived.	
			10^{-6}	Died in 31 hrs.	Typical. Type D in heart's blood.
51	2 (All passages from 25th to 51st at 2 day intervals.)	D + G; G greatly preponderates.	10^{-6}	Survived.	
			10^{-5}	"	
			10^{-4}	Died in 36 hrs.	Typical. Type D in heart's blood.
			10^{-3}	Survived.	
			10^{-2}	Died in 36 hrs.	Typical. Type D in heart's blood.
			10^{-1}	" " 36 "	Typical. Type D and few Type G in heart's blood.
56	5 (All passages from 51st to 56th at 5 day intervals.)	G only.	10^{-6}	Survived.	
			10^{-4}	"	
			10^{-3}	"	
			10^{-2}	"	
			10^{-1}	"	

* All rabbits 600 to 650 gm. in weight. Cultures 6 hour, plain broth.

1.0 cc. of a whole culture of Type G has been injected. On the other hand, rabbits of 600 gm. weight at times are fatally infected by 0.5 cc., more often by 1.0 cc. of undiluted serum broth culture.

Experiment 5. Increase of the Virulence of Type G by Passage through Rabbits.—
Microbe G was isolated from Strain R15-D and carried for thirty-eight passages
in serum broth. It was then tested in the usual manner for virulence on rabbits
of 600 gm. weight. Three rabbits of this series, injected with large doses, suc-
cumbed to typical infections indistinguishable from those caused by Type D.
Pure Type G cultures were isolated in all cases. One of these was carried for
five passages in serum broth and then retested for virulence.' A strain of Type G,
isolated from rabbits succumbing to the second virulence test, was carried in
serum broth for fifteen additional passages, daily transplant, and again subjected
to virulence test. The results are summarized in Table V.

The results summarized in Table V indicate that the virulence of
Microbe G can be raised by passage. While the virulence undoubt-
edly increases, the other characteristics differentiating Type G from
Type D persist and even intensify. This is especially true of the
granular growth character in fluid medium. This becomes very
intense upon animal passage, the organisms of serum broth cultures
clumping into solid masses that fail to break up even after prolonged
shaking of the culture tube. The acid agglutination zone also
broadens considerably. Thus in the case of one of these intensely
granular strains, a four times washed suspension of Microbe G
agglutinated completely in all the tubes of an acetic acid-sodium
acetate buffer series, from pH 5.6 to 3.0.

The experiment demonstrates that granular growth character and
lack of virulence do not necessarily always occur concomitantly. It
had been supposed that there might be some causal connection between
the instability of the organisms and their lack of virulence, but this
idea would seem to be ruled out by Experiment 5.

It will be observed that in the case of the last animal of the series
in Table V some Type D colonies were observed on the serum plates
streaked from the pleural fluid at necropsy. Do these represent
reversion of Type G to the primordial D form? It is impossible to
say whether or not this is the case. This is true because rabbits in
many cases harbor Type D normally. The Type D discovered at
necropsy might, therefore, simply represent individuals which have
invaded the pleural cavity when the animal is already nearly dead
from a true Type G infection. It is this tendency of normal rabbits
to carry Microbe D that makes it unfeasible to attempt the reversion
of Type G to the parent D variety. It is certainly true that Type G

TABLE V.

Increase of the Virulence of Type G by Passage through Rabbits.

Strain.	No. of passages.	Weight of rabbit.	Amount injected intrapleurally.	Result.	Necropsy.
		gm.	*cc.*		
R15-G	0	600	0.1	Survived.	
		610	0.5	Died in 24 hrs.	No necropsy.
		600	1.0	Survived.	
		600	2.0	Died in 32 hrs.	Typical fibrinopurulent pleuritis, pericarditis, and bronchopneumonia.
		600	3.0	" " 5 days.	Pure culture Type G recovered from heart's blood.
R15-G	1	620	10^{-3}	" " 16 hrs.	Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy.
		610	10^{-2}	" " 16 "	Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy.
		610	10^{-1}	" " 19 "	Beginning bronchopneumonia and pleuritis. Type G found in heart's blood at necropsy.
		600	5×10^{-1}	" " 16 "	Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy.
R15-G	2	700	10^{-6}	Survived.	
		750	10^{-4}	Died in 24 hrs.	Lungs congested; pleural fluid increased. Heart's blood pure Type G.
		725	10^{-2}	" " 36 "	
		700	10^{-1}	" " 15 "	Lungs congested. Type G + *few Type D* found in heart's blood.

is by itself able to cause fatal infections when injected in overwhelming dose. This was the case in all of the other animals whose necropsies are recorded in the table.

What is the cause of the loss of the property of invasibility in the case of Type G? It was considered possible that Types D and G

might differ in degree of phagocytability or in their aggressive action against white blood cells.   This idea was put to test in Experiment 6.

Experiment 6. Intrapleural Reaction to the Injection of Large Amounts of Types D and G.—9 hour serum broth cultures of Microbes D and G were carefully shaken up.   1 cc. of each of these was added to 1.5 cc. of plain broth.   The suspensions of Type D and of Type G (total volume 2.5 cc.) respectively were injected intrapleurally into each of two rabbits of 1,600 gm. weight.   Pleural fluid was aspirated after 1, 2, and 4 hours, and smears were made of the withdrawn fluid and stained with Löffler's methylene blue.   The results of this experiment are given in Table VI.

TABLE VI.

Intrapleural Reaction to the Injection of Large Amounts of Types D and G.

Interval before examination.	Microbe D.	Microbe G.
	9 hr. serum broth culture intrapleurally in 1,700 gm. rabbit.	9 hr. serum broth culture intrapleurally in 1,700 gm. rabbit.
hrs.		
1	No fluid obtainable.	No fluid obtainable.
2	Large amount of fluid; myriads of typical bipolar organisms; no slight phagocytes.	Small amount of fluid; no phagocytes; a few clumps of typical bipolar organisms.
4	Myriads of typical organisms; few phagocytes, many badly damaged; slight phagocytosis.	Many phagocytes with ingested organisms; no free organisms observed.
16	Dead; myriads of organisms; some phagocytosis.	No free organisms; few phagocytes. Survived.

It would appear from the experiment just described that Type D possesses a strong antagonistic effect upon the phagocytes, which allows the microbes to gain their primary foothold in the pleural cavity and undergo intense multiplication.   The mutant Type G organisms appear to have lost this aggressive activity and are rapidly clumped and ingested by the white blood cells, which appear at the injection site within 4 hours after inoculation.   These facts would seem to furnish a clue for the study of possible aggressive substances possessed by Microbe D.

It has just been shown that Type G is rapidly disposed of when injected intrapleurally.   It was interesting to öbserve that a culture of Type G which produced no perceptible effect when injected into

rabbits intrapleurally in dose of 1.0 cc., gave rise to abscesses when injected subcutaneously in 0.1 cc. amount. These abscesses were sharply circumscribed, purulent, and teeming with typical G microbes. In no case did the organisms generalize and death result. Type G could be recovered from them for several weeks. This demonstrates a vicariously greater susceptibility to infection depending upon the route of injection. It is well known that phagocytes are more rapidly mobilized on serous surfaces than subcutaneously, and in the light of Experiment 6, the phenomenon of greater infectivity of Type G by subcutaneous route would seem to be due to greater time necessary to bring the phagocytes into play in this region.

<div align="center">DISCUSSION.</div>

The importance of the foregoing observations and experiments is threefold.

1. Various authors have observed the appearance of granular growing varieties in cultures of various microorganisms. Such types have been reported by Arkwright (5) and by Zoeller (6) for *Bacillus dysenteriæ* Shiga. They have been noted in the case of the hog-cholera bacillus by Krumwiede and Provost[1] and by Krumwiede and Valentine for pneumococcus.[2] This phenomenon is not therefore confined to cultures of the rabbit septicemia bacillus and may be found to be of general occurrence. It would be of interest to observe whether lack of virulence is associated with granular growth in these instances.

2. The occurrence, side by side, of the two varieties, the parent Type D, highly virulent, and its mutant Type G, of low invasibility, furnishes an excellent opportunity for the study of the mechanism of virulence. It is possible to investigate the products of secretion and of cell destruction of the virulent Type D, with a view to discovering whether these substances, when added to Type G, might increase its power of invasion. In brief, Type G may be used as a reagent with which to attempt the discovery of the factors responsible for the high invasive power of Type D.

[1] Personal communication.
[2] Personal communication.

3. It is important to find out whether the G forms appear only in strains which have been artificially cultured for some time outside the animal body, or whether they may occur naturally in the animal as well as in the test-tube. This question would seem to have a direct bearing upon the problem of epidemiology. It is at present under investigation and certain results have already been obtained, which will be reported in the near future.

SUMMARY AND CONCLUSIONS.

Type D, bacillus of rabbit septicemia, exhibits marked fixity of the character of virulence. This is true for cultures that are regularly transplanted on serum agar or in broth. It is also to be observed when organisms of this variety are subjected to unfavorable conditions; *e.g.*, remaining for $9\frac{1}{2}$ days without transplant in plain broth. Under such conditions no decrease in virulence was observed. G forms which arose in the same culture during this time exhibited characteristic lack of invasibility.

Different individuals of a given culture of Type D do not vary to a noticeable extent in virulence. This was ascertained by test of virulence of cultures arising from six individuals fished from Type D cultures by Barber pipette.

A Type D culture subjected to passage in plain broth undergoes D → G mutation. Type D and G individuals can be demonstrated to be present in the same culture. The virulence of such a mixed culture is proportional to the relative member of Microbes D and G present. This throws light on certain mechanisms of attenuation.

The virulence of Type G can be raised to a considerable titer by animal passage. Such organisms do not, however, lose their characteristic of granular growth. This last, on the other hand, appears to intensify by animal passage. The acid agglutination zone of Type G strains which have been passed through animals shows a marked broadening.

Microbe D owes its superior invasive power at least in part to its antiphagocytic activity, a property apparently not possessed by Microbe G.

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COLLODION SACS FOR AEROBIC AND ANAEROBIC BACTERIAL CULTIVATION.

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PLATES 53 AND 54.

Among the various media for bacterial cultivation, one that has proved especially valuable for the isolation and maintenance of highly parasitic organisms is a combination of ascitic fluid or dilute blood serum and a fragment of fresh animal tissue. Such a medium was suggested by Theobald Smith[1] and has been extensively developed and used by Noguchi[2] in the cultivation of spirochetes, the globoid bodies of poliomyelitis, and other organisms whose isolation in pure culture he has reported from time to time. *Bacterium pneumosintes,*[3] recovered by Olitsky in 1918 from the filtered nasopharyngeal secretions of patients with epidemic influenza, was isolated under strictly anaerobic conditions in a combination of sterile human ascitic fluid and fresh rabbit kidney tissue.

A certain disadvantage of the medium is found in the presence of protein precipitate derived from autolysis of the tissue fragment. Both macroscopically and in stained specimens this precipitate may obscure the view and either simulate or mask the presence of significant bodies. This disadvantage is apparent especially in the search for hitherto undiscovered microbes and in dealing with filter-passing organisms such as the globoid bodies of poliomyelitis or *Bacterium pneumosintes,* which are just within the limits of vision and are made out with difficulty in early generations. Furthermore, the antigenic protein precipitate makes tissue cultures unsuitable for the production

[1] Smith, T., *J. Boston Soc. Med. Sc.*, 1898–99, iii, 340.

[2] Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

of immune serum or for serological studies in general, because it gives rise to unspecific reactions and so complicates the results.

In the course of recent studies on *Bacterium pneumosintes*, it became desirable to cultivate the organism in a medium without antigenic properties and free from the confusing protein cloud. For this purpose it was proposed to confine the tissue medium in a collodion sac surrounded by distilled water or salt solution, with the prospect that the nutritive and growth-promoting substances of the medium would diffuse through the sac wall and support growth in the surrounding fluid. The design of the apparatus was limited by the necessity for oxygen exclusion and for adjustments of osmotic pressure, so that the collodion sacs prepared for intraperitoneal implantation[4] were found to be unsuitable. It was also desirable that the dialysate cultures might be made in considerable numbers and be subject to frequent examination without disturbance of their anaerobic state. These conditions were met in the method to be described. The apparatus is adapted to various problems in bacterial growth and metabolism, and may therefore find a wider application than we originally intended. Two designs of apparatus have been developed, one containing about 10 cc. of culture medium for routine cultivation, the other holding 50 cc. for mass cultures. The principles involved are the same, but differences in preparation necessitate separate descriptions.

Collodion Sacs in V-Tubes.

For small amounts of culture media a V-shaped glass tube[5] similar to the Smith fermentation tube, except that both ends are open, incloses the collodion sac and its surrounding fluid. The sac almost fills one limb of the V, reaching to the bend at the bottom, and is surrounded by the dialysate fluid, which rises a short distance in the other limb and is thus accessible from its open end. The mouth of the sac is shrunk onto a glass tube sealed into the V-tube by a rubber collar (Text-fig. 1). For anaerobic cultivation the medium in the sac and the dialysate fluid are layered with vaseline, or the V-tube may be placed in an anaerobic jar.

[4] Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.
[5] Kimble Glass Co., Vineland, N. J., or Corning Glass Works (Pyrex), Corning, N. Y.
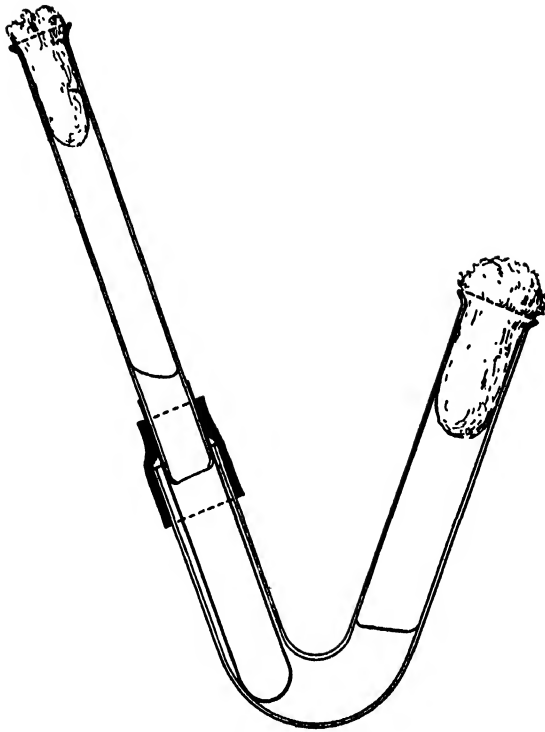
Text-Fig. 1. Cross-section of a collodion sac in its V-tube ready for sterilization.

Preparation of the Collodion Sacs.

The preparation of a considerable number of identical collodion sacs in the simplest and most mechanical manner involves the assembly of some accessory apparatus which adds greatly to the ease and rapidity of the method.

The molds in which the sacs are formed are clusters of five short round bottom test-tubes 10 by 1.4 cm. inside, thrust to the lip through holes in a large (No. 13) rubber stopper (Fig. 1). This stopper fits the mouths of three cylindrical museum jars, 5.5 by 15 cm., provided with glass tops, gaskets, and clamps.[6] One jar contains a 10 per cent solution of gelatin, preserved with 0.3 per cent tricresol; the second contains a thick collodion solution; the third is partly filled with alcohol.

[6] Whitall Tatum Co., New York, N. Y., Fig. 2600, No. 2.

For the purpose of drying the gelatin coating and the collodion membrane with which the molds are lined, we have set up an apparatus to blow air into the molds.   Short stubs of glass tubing are thrust through a No. 13 rubber stopper in a pentagonal pattern corresponding to the molds.   This stopper is set in a small glass funnel for the delivery of air, and the funnel and stopper are supported in a ring clamp and held in place with adhesive plaster.   A second ring clamp supports the rubber stopper containing the cluster of molds above the glass tips, so that a jet of air may be directed into each.

The molds are first lined with a thin coat of gelatin, which prevents the collodion membrane from adhering to the glass.   The ease with which the completed sacs are withdrawn from the molds justifies this preliminary procedure.   To line the molds, which must be clean and smooth, the gelatin solution is melted in a bath of warm water, the cluster of molds is fitted to the mouth of the jar, and the jar is momentarily inverted.   The excess gelatin drains back into the jar and any bubbles in the gelatin break while the film is cooling.   This thin coat of gelatin dries in a few minutes over the air jets described above. A number of mold clusters are prepared before the collodion sacs are made.

The collodion solution should be so thick and viscous that a membrane of sufficient thickness and strength will adhere to the mold even after 3 to 5 minutes drainage.   Such a thick collodion is prepared most easily by evaporation of a thinner solution in a partial vacuum with gentle heat, as described in a former paper.[4]

To form the collodion membranes, a cluster of molds is fitted to the museum jar containing collodion, the jar is inverted momentarily, and the excess liquid is allowed to drain back into the jar until drops no longer form and fall.   It may be necessary to hold the stopper tightly in place during the inversion, as evaporation of the solvents produces a slight pressure in the jar.   A long drainage produces walls of more uniform thickness and allows even minute bubbles in the membranes to drain out or to break.

The collodion-coated molds are then removed from the jar, which is quickly covered again, and are held over the air jets for a few seconds until the ether vapor is washed out and the collodion sets.   As was pointed out in the former paper,[4] the membranes must not be allowed

to dry completely if their permeability is to be retained after heat sterilization. As soon as the collodion sets, the molds are filled with alcohol by inversion of the third museum jar. Here they are allowed to soak for about 10 minutes, during which time the jar may be reversed two or three times to bring fresh alcohol into the tubes.

The molds are then rinsed out several times in cold tap water and immersed for a few minutes while the water penetrates the membranes and softens the gelatin beneath. During this immersion in cold water the lips of the tubes are scraped with a knife to cut the sacs free from the layer of collodion on the rubber stopper and each sac is fitted with the glass tube which is to support it. These glass tubes, 16 by 1.3 cm. outside, with an elastic band snapped on 2 cm. from one end, are of such a size in relation to the molds that they will just slip down into the sacs until checked by the elastic band.

When the gelatin lining under the collodion has softened, the molds with the glass tubes surmounting them are lowered for a minute or two into boiling water in a beaker or water bath. A wire hook (Fig. 1) which engages a small staple centrally placed among the lips of the molds is convenient for this purpose. The hot water shrinks the sacs down tightly upon the glass tubes, melts the gelatin, and so frees the sacs from the molds, so that when the tubes are withdrawn, the sacs come with them. The elastic bands are slipped down over the upper edge of the sacs, holding them in place securely.

The sacs are then rinsed in warm water to remove any traces of gelatin. When empty, they may be tested for leaks by inflation under water. They are now ready for insertion into the V-tubes. These tubes, of 1.5 cm. internal diameter, have limbs 6 and 12 cm. long. A wet collar of pure gum rubber tubing is slipped onto the shorter limb so that it does not obstruct the open end, into which the sac is thrust until its glass-protected neck is in the opening and its closed end has reached the curve at the bottom of the V-tube. The collar is then slipped up to hug the neck of the sac, which is allowed to project a millimeter or two above the collar. Thus the sac is held in place and the inner and outer chambers of the apparatus are sealed (Text-fig. 1). If rubber tubing of the proper size is not available, the collar may be made of a piece of thin rubber dam, 15 by 5 cm., wrapped tightly around the juncture of the V-tube and the sac, and secured with rubber bands.

The sac is then filled and surrounded with distilled water or saline solution, the ends of the tubes are plugged with cotton, and the completed apparatus is sterilized in the autoclave. Finally, the junction between rubber collar, sac, and tube is painted with hot paraffin wax. The V-tubes are carried in test-tube racks cut with slots instead of round holes.

Collodion Sacs in Flasks.

For mass cultures, collodion sacs of about 50 cc. capacity have been prepared. The molds are 50 cc. Erlenmeyer flasks, lined with a gelatin film. The melted gelatin is poured from one flask to another until all are coated. They are drained mouth downward in a rack (Fig. 2), until the gelatin cools and sets. The film is then dried over a jet of air. Collodion is kept in 120 cc. wide mouth rubber-stoppered bottles, into which the filled flasks are allowed to drain until all bubbles have broken and the excess collodion has dripped away. The subsequent treatment is the same as with the smaller sacs. Rather larger glass tubes, 16 by 1.6 cm., are required to support the sacs, and the elastic bands must be stretched tightly to prevent leaks between membrane and tube. The band is put on by collapsing the sac in a 50 cc. centrifuge tube or wide test-tube, around which the rubber band has been tightly doubled and wound. The neck of the sac, with the glass tube in position, projects from the centrifuge tube. The rubber band is then snapped off into place.

As containers, we have had glass tubes 12 by 1.5 cm. fused into 100 cc. Pyrex Erlenmeyer flasks near the bottom, thus forming a spout which gives access to the space surrounding the sac. The sac is collapsed, inserted into the flask, and sealed in place by an elastic collar of tubing or rubber dam. The necks of sacs made in ordinary 50 cc. flasks are not long enough to extend above this collar, hence the necessity for a tight rubber band around the neck. We have had no leakages which could be traced to this source. As with the smaller sacs, the apparatus is partly filled with distilled water or salt solution, plugged with cotton, and autoclaved. The juncture of collar and tube is then paraffined.

Additional Notes on the Preparation and Use of the Sacs.

In order not to prolong the foregoing descriptions, a number of important steps in the preparation of the sacs were not emphasized or discussed. Most important among these steps are those that determine permeability. This subject was treated at some length. in the former paper on collodion sacs, and will not be reiterated here. But attention may be called to several facts which have more recently been noted.

Composition of the Collodion.—We have used a commercial collodion,[7] evaporated in a partial vacuum until thick enough to give a strong membrane with a single coating of the molds. A sufficiently high degree of permeability is conferred upon the membranes by immersion in 95 per cent alcohol, as recommended by Brown.[8] Eggerth,[9] who credits Malfitano with a prior observation, has found that the relative amounts of alcohol and ether in the collodion determine permeability, and has described a series of membranes of graded permeability produced by varying their alcoholic content. Eggerth also found that the addition of 10 to 30 per cent of lactic acid to the collodion greatly increases the permeability of the resulting membranes. These membranes are transparent, tough, and elastic, and of such a high degree of permeability that they readily pass the proteins of serum and ascitic fluid. This is a drawback to their use with a tissue medium when a bacterial growth free from foreign protein is desired. By varying the alcoholic content of the collodion, or by the addition of lactic acid, or by immersion of the undried membrane in alcohol solutions of varied concentrations, a wide range of permeabilities may be obtained.

Eggerth observed that alcohol-rich collodions are made more viscous by the addition of water, and that the presence of even traces of moisture causes them slowly to set. We have had the same experience with thick collodion solutions. Only by keeping the collodion dry can a thick solution be maintained in a fluid state.

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[7] Squibb's Contractile Collodion, U. S. P.

[8] Brown, W., *Biochem. J.*, 1915, ix, 591.

[9] Eggerth, A. H., *J. Biol. Chem.*, 1921, xlviii, 203.

Sterilization by Heat.—The shrinkage and the loss in permeability of collodion membranes when subjected to sterilization in the autoclave were discussed in the former paper. This shrinkage indicates a change in plasticity and cohesion under the influence of heat. Membranes immersed in alcohol are highly plastic and will stretch considerably under pressure without breaking. Thus a sac with a capacity of 45 cc. was stretched to a capacity of 77 cc. by an internal pressure of 12 cm. of 95 per cent alcohol, when its further expansion was checked by the container. On replacement of the alcohol by water, collodion membranes become more rigid, so that water pressures up to the breaking point may be withstood without change in size. If the membranes are heated, however, their plasticity is increased, and the shape that a sac assumes is the result of a balance between the forces of cohesion and the pressure applied to the sac wall. At atmospheric pressure the membrane shrinks progressively as the temperature is raised, but this tendency may be counteracted by the application of pressure within the sac. A slight pressure prevents shrinkage; a greater pressure expands the sac just as it does a sac immersed in alcohol at room temperature. The following experiment illustrates this fact.

Experiment 1. Effect of Pressure on Heated Collodion Membranes. Sac A, Unheated Control.—Capacity 54.8 cc. Immersed in water at room temperature under an internal pressure of 30 cm. of water. After 15 minutes at this pressure, the capacity of the sac was still 54.8 cc. showing that the elastic limit of the membrane had not been exceeded.

Sac B, Heated at Atmospheric Pressure.—Capacity 59 cc. Immersed in a water bath at 20°C. Temperature raised to boiling over a period of about 9 minutes. Boiled 5 minutes. Capacity after heating 38.5 cc.

Sac C, Heated under an Internal Pressure of 25 Cm. of Water.—Immersed in a water bath at 20°C. (with Sac B). Temperature raised to boiling over a period of about 9 minutes. Boiled 5 minutes. Capacity after heating 75.5 cc.

The appearance of these sacs after the experiment is shown in Fig. 3. The relative plasticity of Sacs B and C during the heat treatment is further illustrated by the pear shape they have assumed. Sac A retains the shape of the mold in which it was made.

When a membrane has once been heated, it becomes permanently less plastic and subsequent reheating does not alter its shape and size to a similar extent.

Experiment 2. Effect of Pressure on Reheated Collodion Membranes.—Sac A had a capacity before heating of 52.7 cc. This sac was heated at atmospheric pressure and boiled in a water bath for 9 minutes. After heating, its capacity was 38.7 cc. The sac was again heated under an internal pressure of 32 cm. of water, and boiled for 5 minutes. The capacity of the sac was then 44 cc.

Sac B, capacity 53.5 cc., was boiled under a pressure of 32 cm. of water. The capacity of the sac increased to 68.5 cc. Then the sac was boiled for 5 minutes at atmospheric pressure. Its capacity was reduced to 64.5 cc.

If the shrinkage which occurs in the autoclave is undesirable, it may readily be prevented by sterilization under a slight internal pressure produced by filling the glass neck of the sac with water to a predetermined level, or by a preliminary immersion of the sac in boiling water under a similar water pressure. As tested by the rate of filtration of water under 25 cm. pressure, however, very little difference in permeability results from the prevention of shrinkage, or the expansion of the sac during sterilization by heat. Since it has been possible to prepare sacs which are too permeable for our purpose, the loss in permeability which an undried sac undergoes on sterilization has not been a drawback. Excessive shrinkage due to high temperature is to be avoided, however. We autoclave our sacs for 1 hour at 108°C. (5 pounds steam pressure).

Mode of Use of the Sacs.—The method of filling the sacs and the various uses to which they may be put hardly need a detailed description. Various applications will suggest themselves to meet individual requirements. The apparatus is convenient for either aerobic or anaerobic cultivation. For aerobes the space around the sac should not be entirely filled with fluid. For anaerobes, all bubbles should be tipped out of this space, and both limbs must be sealed with vaseline, or the cultures placed in an anaerobic jar. The tubes or flasks may be inoculated immediately, but we have found it advisable to incubate them first for 24 to 48 hours, to prove their sterility and to permit dialysis of nutrient materials. During this period anaerobic conditions will develop under a vaseline seal. It may be pointed out in this connection that while a seal of vaseline excludes oxygen, it also prevents the free escape of carbon dioxide from the medium. Retention of carbon dioxide may lead to a more rapid acidification of the medium than would otherwise occur.

Hydrogen Ion Concentration of the Dialysate.—In general, the dialy-sate acquires the hydrogen ion concentration of the medium within the sac. In a well buffered colloidal medium, however, the d ffusion of substances which change the hydrogen ion concentration is relatively slow, and consequently the acid produced in, the vicinity of a tissue fragment may not be neutralized by a supernatant column of alkaline medium, and an undue proportion of it may diffuse through the sac wall and acidify the dialysate. We have occasionally found it de-sirable, therefore, to buffer the dialysate or to bring it to a faint alka-linity with sodium hydroxide before inoculation. Phosphate mixtures in the absence of protein appear to be toxic to some bacteria. Peptone broth, when its use is not contraindicated, is an effective buffer and has been used both as a diluent for serum in the medium, and as a dialysate fluid. The addition of dextrose, 1 to 2 per cent, hastens the establishment of anaerobic conditions, and of course increases the nutritive value of the medium for organisms which can utilize it.

Osmotic Pressure.—The osmotic pressure developed in the system depends upon the permeability of the sac in relation to the compo-sition of the culture medium. When a medium containing substances in solution that will not pass through the sac is dialyzed, osmotic pres-sure adjustments are made automatically by imbibition of water and a rise in the level of the medium within the sac. Thus in Fig. 4 the difference in the levels of medium and dialysate represents the osmotic pressure of a mixture of horse serum and dextrose broth dialyzed against distilled water. Imbibition of water may be prevented by filling the sac and its glass neck approximately to the osmotic pressure level, as found by experience with the given medium. The neck of the sac may be filled beyond the pressure level. Then a filtration of fluid from the sac into the surrounding liquid results that may aid the diffusion of nutritive substances. When collodion containing 10 per cent of lactic acid is used, and the sacs are treated with 95 per cent alcohol (also containing lactic acid), their permeability is such that serum proteins pass through freely, and no difference in levels is es-tablished (Fig. 5). Sometimes changes in level break a vaseline seal which is to be restored by gentle heating. .

Symbiosis.—Eggerth[9] found that *Bacillus influenzæ* Pfeiffer could be grown in plain broth in a sac surrounded by a living culture of staph-

ylococcus, streptococcus, or pneumococcus. We have had a similar experience with *Bacterium pneumosintes*. *Bacillus subtilis* was grown in dextrose broth .within collodion sacs. After 24 hours the growth was checked and anaerobic conditions were established by the addition of a vaseline seal. *Bacterium pneumosintes* then grew luxuriantly in dextrose broth surrounding the sacs, although it failed to grow in the same medium in control tubes without *Bacillus subtilis*.

It appears, therefore, that the growth-promoting substances, presumably catalytic or enzymatic in nature, which are necessary to the multiplication of microorganisms may diffuse through collodion membranes of high permeability.

Primary Isolation of Highly Parasitic Organisms.—What little experience we have had indicates that the Smith-Noguchi ascitic fluid-tissue culture is a more favorable medium for the primary cultivation of highly parasitic organisms than is the dialysate of such a medium obtained with collodion sacs. When growth on an artificial medium is once established, however, subplants of *Treponema pallidum*, *Treponema microdentium*, and *Bacterium pneumosintes* have grown readily in the dialysate fluid. The absence of precipitate from the medium permits the collection of a sediment for antigenic and serological purposes composed of the microbic bodies alone. In the case of *Bacterium pneumosintes*, this has made possible a study of its antigenic character.

SUMMARY.

A simple and convenient method is described for the preparation and use of collodion sacs for aerobic and anaerobic bacterial cultivation *in vitro*.

The sacs are suitable for the study of various problems in bacterial growth and metabolism. By their use with the Smith-Noguchi tissue medium, certain microorganisms may be grown in the absence of the confusing, antigenic protein precipitate characteristic of cultures containing fresh tissue, and thus the organisms are obtained in a suitable condition for serological study.

EXPLANATION OF PLATES.

PLATE 53.

FIG. 1. Solutions of collodion, gelatin, and alcohol in museum jars. Cluster of molds inserted in a rubber stopper which fits the jars. 50 cc. Erlenmeyer flask lined with a gelatin film. Hook for lowering molds into boiling water.

FIG. 2. Rack for draining and drying Erlenmeyer flasks lined with gelatin.

PLATE 54.

FIG. 3. Sacs described in Experiment 1. Sac A, unheated control; Sac B, heated under atmospheric pressure; Sac C, heated under an internal pressure of 25 cm. of water. Note the pear shape assumed by Sacs B and C.

FIG. 4. Sac system in a V-tube, containing a kidney tissue medium *versus* distilled water. Both liquids are layered with vaseline. The difference in levels indicates the osmotic pressure of a mixture of horse serum and dextrose broth.

FIG. 5. Flask A, a culture of *Treponema pallidum* in the dialysate of an ascitic fluid-dextrose broth-kidney tissue medium. The microorganisms do not cloud the fluid, but grow in feathery gray flocculi at the bottom of the flask. Flask B, a culture of *Bacterium pneumosintes* in the dialysate of a horse serum-dextrose broth-kidney tissue medium. As shown by the faint image of the string in the tube, the fluid is heavily clouded. These two sacs, made with lactic acid collodion, are so permeable that the level of the medium is not raised by osmotic pressure.
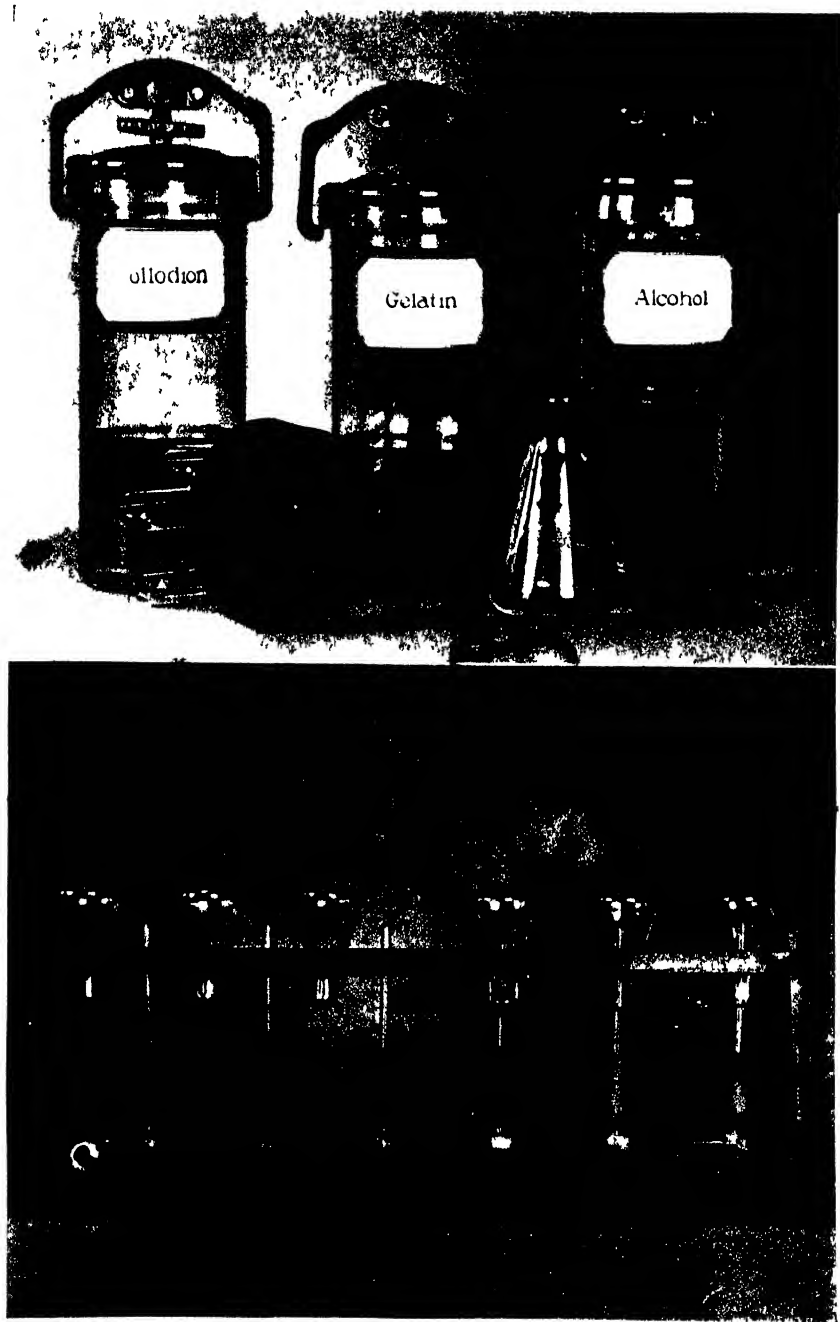
(Gates: Collodion sacs for bacterial cultivation)

STUDY OF THE ACTION OF FOUR AROMATIC CINCHONA DERIVATIVES ON PNEUMOCOCCUS. A COM-PARISON WITH OPTOCHIN.

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Since Morgenroth[1] introduced ethylhydrocupreine (optochin) as a chemotherapeutic agent against the pneumococcus, a number of related compounds has been investigated in the effort to find one more distinctly monotropic. However, up to the present, optochin holds a unique position in that it alone has been proven to influence beneficially an experimental pneumococcus infection in mice, in which animal the bacteriotropism is greater than the coincident organotropism. But this difference in the balance between organotropic and bacteriotropic action of optochin is insufficient to establish a definite therapeutic effect once the pneumococcus infection has become systemic in nature. The use of optochin against pneumococcus infection in man has had a rather comprehensive trial. In pneumonia, the results of investigations of Moore and Chesney,[2] Manliu,[3] Wright,[4] Parkinson,[5] and others have shown its use in this connection to be of little value. This rather discouraging outcome has shown itself to be true, despite the increase in the bactericidal power of the patient's blood (Moore[2] and Wright[4]) and the negligible change in opsonic index (Wright[4]) following its use. Bier[6] has claimed therapeutic effect in treatment of superficial wounds. Uncertain results in pneumococcus meningitis of man were reported by Wolff and Lehmann,[7] Lippman,[8] and Rosenow,[9] while Cordua[10] has found a favorable action in cases of

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[1] Morgenroth, J., and Levy, R., *Berl. klin. Woch.*, 1911, xlviii, 1560, 1979. Morgenroth, J., *Naturwissenschaften*, 1913, i, 609. Morgenroth, J., and Bieling, R., *Berl. klin. Woch.*, 1917, liv, 723.

[2] Moore, H. F., and Chesney, A. M., *Arch. Int. Med.*, 1917, xix, 611; 1918, xxi, 659.

[3] Manliu, J., *Berl. klin. Woch.*, 1916, liii, 58.

[4] Wright, A. E., *Lancet*, 1912, ii, 1633, 1701.

[5] Parkinson, C., *Z. Chemotherap., Orig.*, 1913, ii, 1.

[6] Bier, A., *Berl. klin. Woch.*, 1917, liv, 717.

[7] Wolff, S., and Lehmann, W., *Jahrb. Kinderheilk.*, 1914, lxxx, 188.

[8] Lippman, *Berl. klin. Woch.*, 1917, liv, 781.

[9] Rosenow, G., *Deutsch. med. Woch.*, 1920, xlvi, 9.

[10] Cordua, R., *Berl. klin. Woch.*, 1921, xlviii, 1323.

meningococcus meningitis. However, Kolmer and Idzumi[11] were unable to produce a favorable influence on an experimental pneumococcus infection of the meninges of dogs and rabbits.

The realization of the limitations of this compound led us to investigate the cinchona derivatives synthesized and reported by Jacobs and Heidelberger.[12] It seemed to us that regardless of whether or not a substance was found that possessed greater activity than optochin, a biological study of these numerous compounds—so closely related to optochin—might be an aid in further chemical and biological investigations.

It was found at the outset that certain of these modified cinchona compounds possess a remarkably high bactericidal activity for pneumococci *in vivo*. In this paper we wish to present a study of four active chemicals in comparison with optochin. These four were chosen, as is shown below, for the reason that they represent a uniform series of hydroquinine derivatives, thus affording opportunity for a comparison between the chemical constitution and biological activity of closely related compounds.

To show clearly the relationship between these compounds and the exact chemical differences, structural formulas are given in Text-fig. 1. The first change in hydroquinine is the addition of chloracetamide on the quinuclidine ring, making hydroquinine chloracetamide. In turn, one of the amide hydrogens of the acetamide group is substituted by 'a benzene nucleus forming hydroquinine chloroacetanilide, the first important member of this series. The remaining drugs are hydroxy substitution products of the latter, in which the OH group is substituted in the para or the meta, or in both para and meta positions in the benzene nucleus, yielding respectively hydroquinine *p*-chloroacetylaminophenol hydrochloride, hydroquinine *m*-chloroacetylaminophenol hydrochloride, and hydroquinine 4-chloroacetylaminopyrocatechol hydrochloride. To avoid repetition of these names in this paper, the laboratory number will be used, as follows: the chloroacetylanilide, C 29; the *p*-chloroacetylaminophenol, C 36; the *m*-chloroacetylaminophenol, C 40; and the 4-chloroacetylaminopyro-

[11] Kolmer, J. A., and Idzumi, G., *J. Infect. Dis.*, 1920, xxvi, 355.
[12] Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1919, xli, 2090.

catechol, C 110.   As can be seen from the chart, these substances are
derived from the hydroquinine and not from the ethylhydrocupreine
nucleus, the only difference in the two compounds being that one is
a methoxy and the other an ethoxy substitution on the quiniline
nucleus.   There is in these chemicals a combination of two bactericidal
compounds—the quinines and benzenes.   Since Lister introduced

Hydroquinine hydrochloride   Hydroquinine chloracetamide

Hydroquinine chloroacetanilide   Hydroquinine   Hydroquinine
                                   p-chloroacetylaminopheno'   m-chloroacetylaminophenol
                                   hydrochloride   hydrochloride

Hydroquinine
4'-chloroacetylaminopyrocatechol   Ethylhydrocupreine hydrochloride
hydrochloride
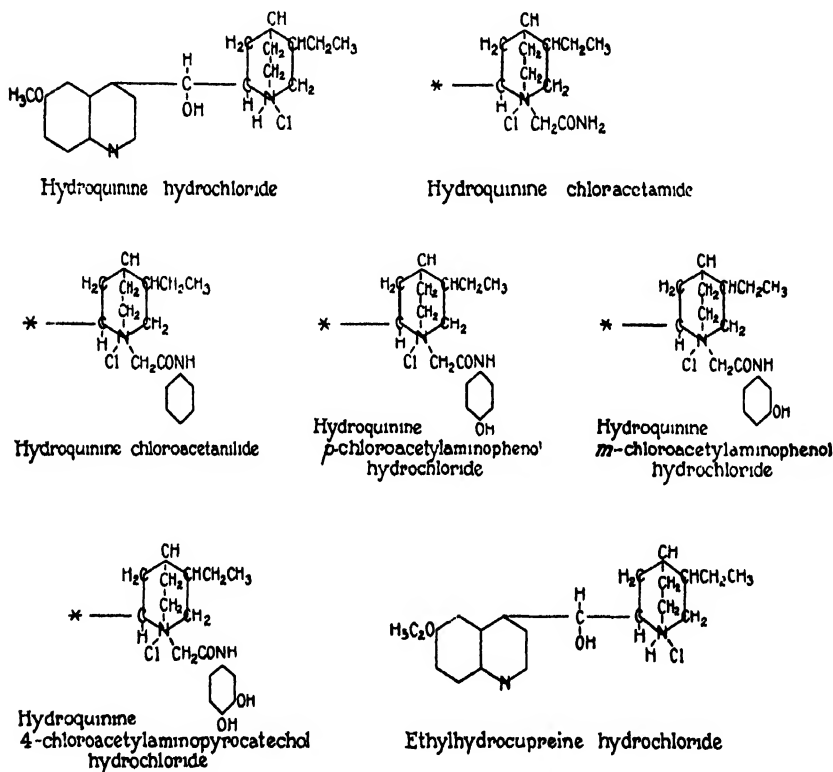
TEXT-FIG. 1.   Structural formulas of the cinchona alkaloids studied.

phenol into antiseptic surgery practically all the hydroxybenzene
compounds have been employed for the same or similar purposes,
but abandoned for one reason or another as impracticable.   These
new organic chemicals can therefore be thought of either as additive
compounds of the benzene derivatives to the hydroquinine nucleus
or the hydroquinine to the benzene nucleus by means of a common
linkage CH₂ONH.

General Procedure.

Since we undertook to make a comparative study of the different chemicals, work was planned so that each experiment, in so far as was practical, embraced all the drugs under the same conditions. Young mice of a healthy stock, weighing from 15 to 18 gm., were employed throughout. Animals of this weight were used because it was early found that large, and especially old, female mice reacted irregularly. Doses of the chemical were approximated to 18 gm. mice. Each mouse was autopsied at death and one loop of heart's blood smeared on a blood agar plate, presence or absence of growth being noted at the end of 24 hours. Animals that survived were kept for 30 days and then killed. Cultures were made from a few animals living the 30 day period, but inasmuch as no pneumococci could be found, this procedure was abandoned. Unless otherwise stated in the tables pneumococci were present at death in the blood stream of all mice recorded. Since we found considerable variation in numbers of organisms in both the treated animals and the controls, little stress was laid on this factor. For each experiment, controls were run in duplicate with what had been found from previous experience to be 1, 10, and 20 M.L.D. This minimum lethal dose was calculated each time for the given experiment after the death of the controls, and was taken to be the smallest number of organisms, or the highest dilution of the culture (made in physiological salt solution), causing the death of duplicate mice in 48 hours.

The chemicals were dissolved in boiling sterile distilled water. The pneumococcus used was a Type I (Neufeld).[13] Its virulence was such that generally two to four organisms (fluctuations occurring from time to time from two to twenty organisms) of a 6 hour culture, as determined by plating, injected into the peritoneal cavity of a mouse, resulted in the death of the animal within 48 hours. The intravenous virulence of this organism was rather low, requiring approximately 200,000 diplococci to cause death, or 10,000 times as many as by the intraperitoneal route.

Solubilities.

In Table I are given the solubilities of the chemicals in water, physiological salt solution, and serum. They have a practical bearing as can be seen below, in view of the bactericidal results obtained *in vivo* and *in vitro*. In making the determinations with water and physiological salt solution, each respectively was added slowly in measured amounts on 100 mg. of substance until solution was obtained. The solution in serum represents the concentration of the drug that does not cause precipitation in the serum, and can hardly be termed solubility. Both the aromatic compounds and optochin

[13] The strain of pneumococcus was kindly furnished by Dr. O. T. Avery.

are protein precipitants.   C 29 is the least and optochin the most soluble, while C 36, the para-hydroxybenzene derivative, is more soluble than C 40, with the OH group in the meta position.   C 110, having the OH group both in the para and meta positions, holds an intermediate position between these two compounds.

TABLE I.

Solubilities.

Chemical.	Percentage of chemical in solvent.		
	Water.	Physiological salt solution.	Horse serum.
C 29	0.3	0.12	0.06
C 36	10.0	10.0	0.12
C 40	2.0	2.0	0.05
C 110	4.0	4.0	0.25
Optochin.	10.0	10.0	0.4

Bactericidal Action in Vitro.

Bactericidal action has been measured in two ways, (*a*) by a constant dose of organisms inoculated into varying concentrations of the chemical and (*b*) by a constant concentration of drug to which were added varying numbers of organisms.   Since Chesney[14] has shown

TABLE II.

*Bactericidal Action in Whole Blood in Vitro.**

Chemical...............................	C 29	C 36	C 40	C 110	Optochin.
Dilution sterile in 2 hrs...........	1:1,500	1:750	1:1,000	1:500	1:8,000

* Neufeld pneumococcus.

that a young culture is more resistant to bactericidal agents than an old one, a 6 hour culture of the Neufeld pneumococcus was employed in these tests, in order that conditions might resemble as nearly as practicable those *in vivo*.   In the first method whole defibrinated blood was used as medium.

[14] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

To 2 cc. of the blood was added the chemical in water solution with concentrations adjusted to give a constant volume in making the following series of dilutions: 1:500, 1:750, 1:1,000, 1:1,500, 1:2,000, 1:4,000, 1:8,000, and 1:12,000. This series was inoculated with 0.2 cc. of a broth culture per tube and incubated for 2 hours in a water bath at 37°C. One loopful from each tube was then distributed over one-fourth of a blood agar plate. These plates were incubated for 96 hours and read. From Table II it can be seen that under these conditions the aromatic derivatives are less active than optochin, the latter being about eight times as potent as C 29, the best of the others.

In the second method, bactericidal activity was determined by exposing the organisms in physiological salt solution, 50 per cent serum, and 50 per cent defibrinated rabbit blood, to the action of the chemicals for 5 minutes.

The different dilutions of pneumococci were made in 0.4 cc. of physiological salt solution, undiluted serum, and whole blood, respectively, by adding 0.1 cc. of the appropriate dilution of the culture to make the final concentration the one desired. The chemicals were dissolved in water, so that 0.5 cc. contained 1 mg. of substance. In the final test (Table III) there was a volume of 1 cc. with 1 mg. of chemical and a series of dilutions of a 6 hour culture of 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000. The controls in each case were made at the end of the experiment, 0.5 cc. of physiological salt solution being added to make the volume up to 1 cc. To each tube containing 0.5 cc. of culture was added 0.5 cc. of the chemical. The mixture was put in the water bath at 37°C. for 5 minutes, and then 0.05 cc. was pipetted into 100 cc. of meat infusion broth (pH 7.4), thus bringing the chemical dilution above its bactericidal potential (1:2,000,000). The flasks were under observation for 4 days and results recorded each 24 hour period except for the test on blood, the first observation in this case being made 12 hours after inoculation. Controls all grew out in 24 hours, while in this length of time no visible growth occurred in the flasks containing organisms subjected to the chemicals. The order of the rapidity of bactericidal action in decreasing ratio is as follows: C 29, C 40, C 36, optochin, and C 110. Neither serum nor blood diminished the activity of C 29 or optochin. The unchanged benzene nucleus derivative possesses the most rapid action; the one with the OH group in the meta position is more active than the one with the OH in the para position; while the compound with the OH group in both meta and para positions is still less active. Optochin in this experiment proves to have a slow rate of activity, as had been found by Solis-Cohen, Kolmer, and Heist [15]

In addition, the dilution of the drugs that inhibited bacterial growth, with resultant death, was determined on meat infusion broth (pH 7.8).

[15] Solis-Cohen, S., Kolmer, J. A., and Heist, G. D., *J. Infect. Dis.*, 1917, xx, 313.

TABLE III.

Bactericidal Action with a 5 Minute Contact Period. 1 Mg. of Chemical Used in Physiological Salt Solution, Serum, and Whole Blood.

Chemical	Dilution of pneumococcus (Neufeld).	Physiological salt solution						50 per cent horse serum						50 per cent defibrinated rabbit blood					
		Chemical				Control		Chemical				Control		Chemical				Control	
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	12 hrs.	24 hrs.
C 29	1:10	0	+	0	0	+	⊹	0	+	0	0	+	+	0	0	+	0	+	+
	1:100	0	+	0	0	+		0	+	0	0	+		0	0	0	0	+	
	1:1,000	0	0	0		+		0	+			+		0	0	0	0	+	
	1:10,000	0	0			+		0	0			+		0	0	0		⊞	
	1:100,000	0	0			⊞		0	0			⊞		0	0	0			
C 36	1:10	0	+	0	0	+	+	0	+	+	+	+	+	+	+			+	+
	1:100	0	+	0	0	+		0	+	0	0	+		+	+			+	
	1:1,000	0	+			+		0	+			+		0				+	
	1:10,000	0	0			+		0	+			+		0				⊞	
	1:100,000	0	0			⊞		0	0			⊞		0					
C 40	1:10	0	+	0	0	+	+	0	+	0	0	+	+	+	+			+	+
	1:100	0	+	0	0	+		0	+	0	0	+		+	+			+	
	1:1,000	0	0	0	0	+		0	+			+		0				+	
	1:10,000	0	0			+		0	0			+		0				⊞	
	1:100,000	0	0			⊞		0	0			⊞		0					
C 110	1:10	0	+	+	0	+	+	0	+	+	0	+	+	+		+		+	+
	1:100	0	+	0		+		0	+	0		+		+		+		+	
	1:1,000	0	+			+		0	+			+		+				+	
	1:10,000	0	0			+		0	0			+		0				⊞	
	1:100,000	0	0			+		0	0			⊞				+			
Optochin.	1:10	0	+	+	0	+	+	0	+	0	0	+	+	+	+	0	+	+	+
	1:100	0	+	0	0	+		0	+	0	0	+		0	0	0	+	+	
	1:1,000	0	+			+		0	+			+		0	0			⊞	
	1:10,000	0	0			+		0	0			⊞		0					
	1:100,000	0	0			+		0	0			⊞		0					

0.1 cc. of a 6 hour culture was pipetted into each tube (10 cc. volume) of broth containing the series of dilutions represented in Table IV. The inoculated broth was incubated for 4 days, and then 1 cc. from the last tube showing no visible growth was plated for confirmation. Hydroquinine (C 9) and hydroquinine chloracetamide (C 20) were added to the experiment for comparison.

It is rather interesting to note that the substitution of the chloracetamide on the hydroquinine nucleus decreases its bactericidal activity. With the exception of optochin, the same relationship persists between this group of chemicals as in the preceding experiment. C 29 is as active as optochin under these conditions.

TABLE IV.

Bactericidal Action in the Presence of Broth (pH 7.8).

Chemical.	Dilution in broth.														
	1:2,500	1:5,000	1:10,000	1:15,000	1:20,000	1:30,000	1:40,000	1:60,000	1:80,000	1:120,000	1:160,000	1:240,000	1:320,000	1:480,000	1:640,000
C 9								+	++	++	++	++	++	++	++
C 20		++	++	++	++	++	++	++	++	++	++	++	++	++	++
C 29												+	++	++	++
C 36								++	++	++	++	++	++	++	++
C 40										++	++	++	++	++	++
C 110				+	++	++	++	+ +	++	++	++	++	++	++	++
Optochin.											++	++	++	++	++

Toxicity.

Instead of the toxic dose the largest tolerant dose is given, as determined by averaging several titrations (Table V). The injections *per os* were made by means of a small silver stomach tube attached to a syringe by flexible rubber tubing, the volume injected always being 0.5 cc. From the standpoint of the largest tolerant dose, the same relative positions of the aromatic compounds exist organotropically as were shown to exist parasitotropically; that is, with these four aromatic compounds toxicity runs parallel with bactericidal power—the most toxic, C 29, is the most bactericidal. If rapidity of bactericidal action is used as the criterion, this is also true of optochin. The intravenous toxicity is perhaps largely a physical phenomenon due to protein precipitation.

In the balance between the organotropism and parasitotropism lies the hope of chemotherapy. It is perhaps unreasonable to suppose that a drug will ever be discovered that is strictly monotropic in its action. Manifestly, all the drugs used in clinical medicine show distinctly a therapeutic dose beyond which they set up complex phenomena causing aggravation rather than amelioration. Just as a drug is active often because of its affinity for a certain monocellular tissue in the animal body, so must we look for a drug that has a greater toxicity for monocellular organisms—bacteria—than for any cells vital to the defensive mechanism of the animal. It is perhaps impossible, at this stage of our knowledge, to make this comparison. However, if the bactericidal action of the largest tolerant dose of these

TABLE V.

Toxicity.

Chemical.	Largest tolerant dose.			
	Intravenous.	Intraperitoneal.	Subcutaneous.	*Per os.*
	mg.	*mg.*	*mg.*	*mg.*
C 29	0.2	0.5	2.0	5.0
C 36	0.5	2.5	5.0	10.0
C 40	0.2	1.5	3.0	8.0
C 110	0.5	4.0	6.0	12.0
Optochin.	0.6	3.0	4.0	10.0

chemicals (Table V) for the experimental animal—the mouse—is compared with the number of virulent pneumococci that are killed *in vitro* (Table III) in as short a time as 5 minutes, the first step of this comparison is taken; that is, a non-toxic dose of the drug (for mice) is capable of killing *in vitro* many multiple lethal doses of virulent organisms. The following experiments were designed to show whether these relationships exist *in vivo*.

We wish to present the results of these *in vivo* experiments from a twofold standpoint. First, will the drugs kill multiple lethal doses of virulent pneumococci when chemical and organism are injected simultaneously into a walled off body cavity, such as the peritoneal? Second, will the drugs diffuse, and influence the experimental infection when injected by routes other than the one by which the animal was

infected? Briefly, is there either a local or a systemic bactericidal action *in vivo*, or both?

Simultaneous Injection of Multiple Lethal Doses of Pneumococci with Varying Amounts of Drugs.

The simplest possible procedure, perhaps, by which to estimate *in vivo* bactericidal activity resulting in an animal following the injection of a given drug is a simultaneous injection of organism and
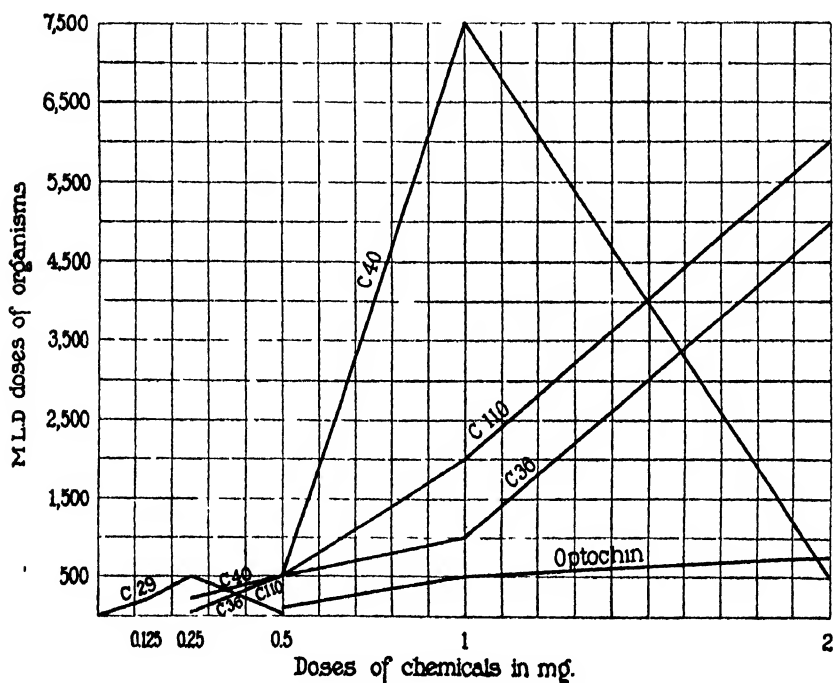
TEXT-FIG. 2. Representation of the number of minimum lethal doses of organisms influenced by different doses of drugs—a therapeutic zone phenomenon.

chemical into the peritoneal cavity. By using as an indicator a microorganism of known virulence, it is possible to obtain within limits of biological accuracy an estimation of the number of organisms killed. The curves depicted in Text-fig. 2 are only approximate, owing to the wide range in dosage of organisms. These curves represent a titration of the number of minimum lethal doses of pneumococci killed by each of four doses of drugs, decreasing by halves from the

largest non-lethal dose. The number of minimum lethal doses of organisms, namely 1, 10, 100, and 1,000, in the preliminary titrations, was the same for all the doses of each chemical. Subsequently dilutions representing minimum lethal doses intermediate between the above doses were injected to furnish data given in Text-fig. 2. All titrations were made in duplicate.

The work with the aromatic compounds gave consistent results throughout. Optochin, on the other hand, varies in its effectiveness, especially in the 2 mg. dose. With this dose, the 740 M.L.D. from which the mice were protected is the average of five titrations: 400, 100, 200, and 1,000 to 2,000 M.L.D. The last two figures were obtained when the pneumococcus was at its greatest virulence, two to four organisms causing the death of the control in 48 hours. At the time of the other three experiments, 20 to 40 organisms were necessary to cause death of the controls in this interval. The fact that approximately ten times as many organisms were present in the unit injected at one time as at the other possibly accounts for the variable results. As the aromatic compounds are more rapid in their action, this may account for the fact that the results are more constant than with the more slowly acting optochin.

Hydroquinine has been shown by Morgenroth, Kolmer,[15] and Tugendreich and Russo[16] to have slight but distinct therapeutic value, though it is not so active as ethylhydrocupreine. By using the same technique as employed with these aromatic derivatives, it was found that hydroquinine protected against 100 M.L.D. at a time when this was represented by approximately 200 pneumococci. Hydroquinine chloracetamide, in the same experiment, protected against only 10 M.L.D. The results with these two chemicals show a parallelism between *in vitro* and *in vivo* bactericidal action (Table IV).

The very striking zonal reaction noted with C 29 and C 40 shows that there is an optimum concentration of the chemicals, below their toxic dose, at which virulent pneumococci are killed *in vivo*. If a therapeutic amount larger or smaller than this is injected, the mice die of septicemia. Manifestly, whatever the mode of action in this case may be, the normal defensive mechanism of the animal

[16] Tugendreich, J., and Russo, C., *Z. Immunitätsforsch.*, *Orig.*, 1913, xix, 156.

is weakened, the relation between mono- and polytropism being reversed. This same phenomenon occurs with the other chemicals, as will.be shown later in this paper.

Comparison of Single and Repeated Injections of the Same Amount of Drug.

To make a still more vigorous test on this local effect, experiments were carried out on single and repeated injections of drugs, 1 and 2 hours after the animal was experimentally infected.

It has been shown by many workers that it is more difficult to cure an infection if time elapses between inoculation of the animal and application of remedial measures. That this is true with these chemicals is shown in Table VI. The experiment thus represented was carried out to show the relative effectiveness between single doses, and the same amount divided into fifths. These two methods of treatment were applied to mice 1 hour after injection of organisms, those receiving a single dose of drug being injected with 10 M.L.D., while those having the dose divided were injected with 10, 20, and 50 M.L.D.

The single dose of the drug proved ineffective, except with optochin, in which case one mouse with 10 M.L.D. survived. The repeated divided dose presented a different picture, as many as 50 M.L.D. of the organisms being killed with the aromatic compounds. Optochin in these small doses was unable to cure an animal infected with more than 10 M.L.D. The drop in the effectiveness of the chemicals when injected 1 hour after the organism is remarkable. The simultaneous procedure with C 40, for instance, proved to be lethal for 8,000 M.L.D. of organisms; injections deferred for 1 hour had no influence on 10 M.L.D. The power of the drug was thus decreased approximately 8,000 times.

Inasmuch as optochin, under these conditions, did not hold up as well as the other derivatives, the possibility was suggested that dosage was at fault. To establish this point and to determine the optimum concentration for five hourly injections, the experiment represented in Table VII was carried out.

2 hours after the injection of the pneumococci, a series of five injections of each doses of the chemicals, at intervals of 1 hour, was begun. The repeated 1 mg. doses of C 36 and C 40 were toxic for the mice. However, at death the C 36 animals had pneumococci in their blood streams, while with C 40, the animal with

TABLE VI.

Comparison of Single and Repeated Divided Doses of Drug, Given 1 Hour after Injection of the Organism.

Length of life.

After single dose of chemical.

Chemical.	Dose.*	No. of mice.	No. of M.L.D.* 10
	mg.		
C 29	0.5	4	24 hrs. 9 days; 16 days; 24 days.
C 36	1.0	4	60 " 72 hrs.; 36 hrs.; 120 hrs.
C 40	1.0	4	24 " 72 " 48 " 60 "
C 110	2.0	4	72 " 240 " 96 " 168 "
Optochin.	2.0	4	72 " 120 " 140 " L.
Controls.	0	6	0.00000001 cc., 48 hrs. = 1 M.L.D.

After repeated doses of chemical, five times at hr. intervals.

Dose.*	No. of mice.	No. of M.L.D.*		
		10	20	50
mg.				
0.1	2	L.; L.†	144 hrs.; L.	L.; L.
0.2	2	" "	L.; L.	" "
0.2	2	" "	" "	" "
0.4	2	" "	" "	" "
0.4	2		48 hrs.; L.	48 hrs.; 48 hrs.
0	6	0.00000001 cc., 48 hrs. = 1 M.L.D.		

* Both drug and organisms were injected intraperitoneally.

† In the tables L. indicates lived.

TABLE VII.

Intraperitoneal Varying Doses of Drug Repeated Hourly for 5 Hours, Beginning 2 Hours after Injection of the Organism Titration of Chemicals.

Chemical.	No. of M.L.D.	No. of mice.	Length of life after varying doses.			
			1.0 mg.	0 5 mg.	0.25 mg.	0.125 mg.
C 29	10	2		36 hrs.; 24 hrs.	36 hrs.; 36 hrs.	10 days; L.
	100	2		24 " 24 "	36 " 36 "	36 hrs.; 36 hrs.
C 36	10	2	24 hrs.; 24 hrs.	36 " L.	L.; L.	
	100	2	24 " 36 "	60 " "	36 hrs.; 96 hrs.	
C 40	10	2	24 " 24 "	36 " 60 hrs.	L.; L.	
	100	2	36 " 24 "	36 " 36 "	60 hrs.; L.	
C 110	10	2	L.; L.	L.; L.	L.; L.	
	100	2	36 hrs.; L.	60 hrs.; L.	36 hrs.; 36 hrs.	
Optochin.	10	2	L.; L.	7 days; L.	48 " 48 "	
	100	2	60 hrs.; L.	36 hrs.; 60 hrs.	36 " 36 "	
Controls	0	4	Succumbed to 0.0000001 cc. = 1 M.L.D.			

TABLE VIII.

Intraperitoneal Doses of Drug Repeated Hourly for 5 Hours, Beginning 2 Hours after Injection of Varying Doses of the Organism.

Titration of Organisms.

Chemical.	Dose.	No. of mice.	Length of life.		
			No. of M.L.D.		
			10	20	40
	mg.				
C 29	1.25	4	L.; L.; L.; 96 hrs.	36 hrs.; 36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.; 36 hrs.
C 36	0.25	4	96 hrs.; 96 hrs.; 96 hrs.; 24 hrs.	L.; L.; L.; L.	36 " 36 " 36 " L.
C 40	0.25	4	L.; L.; L.; L.	96 hrs.; 36 hrs.; 36 hrs.; 36 hrs.	36 " 36 " 36 " 36 hrs.
C 110	0.5	4	36 hrs.; L.; L.; L.	36 " L.; L.; L.	L.; 36 hrs.; L.
Optochin.	1.0	4	36 " " " " "	L.; L.; L.; L.	36 hrs.; 96 hrs.; 96 hrs.

the 10 M.L.D. gave a sterile heart's blood culture, that of the 100 M.L.D. mouse being positive. All other mice of this experiment proved to have a septicemia at death. It can be seen with repeated, as with single injections, of C 29 and C 40, that there is a definite optimum concentration at which these drugs act; in addition, C 110, C 36, and optochin show this zonal phenomenon with repeated doses, C 110 and optochin having a broader zone than the other chemicals. Optochin, however, as in the preceding experiment, did not influence more than 10 M.L.D. when administered in small (0.25 mg.) repeated doses.

In the experiment represented in Table VIII, we estimated the number of minimum lethal doses of pneumococci killed by the optimum concentration of the drug. The results parallel those represented in Table VII, and in addition show that C 36 and C 110 and optochin influence as many as 20 M.L.D. The irregularity seen with C 36 has occurred in other experiments. Often with a small number of organisms, the infection proceeded as in the control mice, while a larger number of organisms was killed.

Diffusion of Drugs.

Thus far our work indicates that within certain definite limits these cinchona compounds have marked *in vivo* bactericidal activity. Drugs to be effective in an infectious disease should have more than this local action. They must be able to penetrate or diffuse throughout the animal body and perhaps in the last analysis maintain a definite concentration in the blood stream. This is especially true with the experimental infection of pneumococci in mice, which is a typical septicemia and in no way comparable to a lobar pneumonia. With this idea in view, the following experiments were carried out.

Subcutaneous Administration of Drugs Following Intraperitoneal and Intravenous Injection of Pneumococci.—This is the method which Morgenroth and Levy[1] and Moore[17] used in their investigations with optochin on mice. Their results show that optochin does diffuse into the circulation, inasmuch as an experimental infection was controlled in its incipiency.

Table IX is a record of three experiments in which the drug was administered subcutaneously, and the organism injected either intraperitoneally or intravenously. In the first experiment duplicate mice were given 1 and 10 M.L.D.

[17] Moore, H. F., *J. Exp. Med.*, 1915, xxii, 269, 389, 551.

TABLE IX.

Subcutaneous Injection of Drug.

Chemical	Organism injected intraperitoneally.			Organism injected intraperitoneally.			Organism injected intravenously.		
	Dose. *mg.*	No. of M.L.D.	Length of life	Dose. *mg.*	No. of M.L.D.	Length of life.	Dose. *mg.*	No. of M.L.D.	Length of life.
C 29	0 5	1 / 10	L; L / 48 hrs.; 36 hrs	2.0	1 / 10	48 hrs.; 48 hrs. / 48 " 48 "	0.5	½ / 1	60 hrs.; 48 hrs. / 48 " 48 "
C 36	1.0	1 / 10	L; L. / 36 hrs.; 36 hrs	4 0	1 / 10	36 " 48 " / 36 " 48 "	1.0	½ / 1	48 " 96 " / 48 " 48 "
C 40	1.0	1 / 10	L; L. / 36 hrs.; 48 hrs.	2.0	1 / 10	L; 48 hrs / 48 hrs.; 48 hrs.	1.0	½ / 1	L.; L. / 60 hrs.; 60 hrs.
C 110	2.0	1 / 10	36 " 48 " / 36 " 36 "	4.0	1 / 10	48 " 48 " / 48 " 48 "	2 0	½ / 1	60 " 48 " / 60 " 60 "
Optochin.	2.0	1 / 10	L; L. / 48 hrs.; L.	4.0	1 / 10	L; L. / " 96 hrs.	2.0	½ / 1	L.; 60 hrs. / 60 hrs.; 48 hrs.

of pneumococci intraperitoneally, followed immediately by the drug. There seem to be indications that the drugs, save C 110, do have a systemic distribution in sufficient quantity to influence the progress of the attack of virulent organisms. This action is slight, however, optochin having greater effectiveness than the other compounds. In the second experiment a large non-toxic dose was given to duplicate mice with 1 and 10 M.L.D. The infection was not influenced, except in the case of optochin. The same zonal phenomenon persisted that occurred with C 29 and C 40 and in fact all the aromatic compounds (Text-fig. 1); that is, a large non-toxic dose of the drug did not favorably influence the course of the infection, the mice, if anything, being more sick than those given smaller doses. The third experiment represents an attempt to influence the course of an experimental septicemia. Again in duplicate, mice were given ⅕ and 1 M.L.D. (1:1,000 and 1:5,000) of a 6 hour culture of Neufeld pneumococci intravenously, followed immediately by the drug subcutaneously. It is seen that not only did the mice injected with 1 M.L.D. of the drug die, but all save those with C 40 and optochin of the ⅕ M L.D. series. Seemingly with this dose, at least, the natural defensive mechanism is interfered with, so that the animal receiving one-fifth of the number of pneumococci (approximately 40,000 organisms) necessary to cause the death of a mouse, died in the same time as the controls.

Intravenous Injection of Drug and Organism; Intravenous Injection of Organism and Intraperitoneal Injection of Drug, and Vice Versa.—If the subcutaneous injection of the drug decreased the ability of the animal to cope with virulent organisms, one might expect that injection of the drug intravenously would also destroy a part of this defensive process.

That this is the case is shown in the first experiment in Table X. Although optochin was not so toxic as the aromatic compounds, a single dose of the drug did not influence the progress of the infection with 1 M.L.D. of organisms. In the next experiment with the intravenous organism and intraperitoneal drug, the outcome was practically the same as with the intravenous injection of the organism and subcutaneous injection of the drug. Again not only does the intraperitoneal injection of the chemical not affect favorably an experimental septicemia in mice, but causes the animal to succumb to a number of organisms below the minimum lethal dose.

Diffusion of Drugs through the Gastrointestinal Tract.—Inasmuch as there has been found to be a definite therapeutic dose for these chemicals when given by the intraperitoneal and intravenous routes, varying amounts were employed to test their diffusibility from the gastrointestinal tract.

TABLE X.

Simultaneous Injection of Drug and Organism.

Chemical	Both drug and organism injected intravenously.			Organism injected intravenously, drug intraperitoneally.			Organism injected intraperitoneally, drug intravenously.		
	Dose.	No. of M.L.D.		Dose.	No. of M.L.D.		Dose.	No. of M.L.D.	
		½	1		½	1		½	1
	mg.			*mg.*			*mg.*		
C 29	0.1	72 hrs.; 144 hrs.	60 hrs.; 11 days.	0.5	36 hrs.; 36 hrs.	36 hrs.; 36 hrs.	0.1	L.; L.	36 hrs.; 36 hrs
C 36	0.2	48 " 120 "	36 " 36 hrs.	1.0	120 " 48 "	36 " 36 "	0.1	" 36 hrs.	36 " 36 "
C 40	0.1	36 " L.	36 " 7 days.	1.0	48 " 60 "	36 " 48 "	0.1	" L.	36 " 36 "
C 110	0.3	L.; L.	60 " 72 hrs.	2.0	L.; L.	60 " 72 "	0.3	" 36 hrs.	36 " 36 "
Optochin.	0.3	" "	120 " 48 "	2.0	" "	120 " 48 "	0.3	" 36 "	36 " 36 "

A series of four doses was used—both single and repeated—each being one-half the amount of the preceding (Table XI). Mice were injected intraperitoneally with 2 to 4 M.L.D. of organisms immediately followed by the drug *per os*. With the single dose there was demonstrated a diffusibility sufficient to influence this small number of pneumococci. As with the intraperitoneal injection of the drug, there is in this case also a concentration of chemicals above which the organisms are not killed, and the animal dies of septicemia. Although C 29 and C 110 delayed death, there were no actual survivals. C 36, C 40, and optochin did prevent the development of an infection, optochin, as before, being active in a large dose. On the other hand, repeated doses of all the drugs exerted a destructive influence upon the organism.

From this experiment it would be very difficult to say which one of the chemicals was the best. To determine this fact, the optimum concentration of the drug was used in another experiment with multiple lethal doses of pneumococci (Table XII).

Three doses of the chemical were given at 2 hour intervals, beginning immediately after the injection of the organisms. Although there was very little variation in effect of the different chemicals, results prove that inasmuch as there were some survivals, some action other than purely local was exerted. In the same table are presented the results concerning the effectiveness of drugs after intravenous injection of the organism. It happens that by this method of administration the same destructive action of the defensive mechanism of the animal does not occur with C 40, C 110, and optochin, as occurred by intraperitoneal route. In fact, with these compounds, there was a survival of the mice injected with 1 M.L.D. This experiment with intravenous injection of the organism was repeated, with multiple minimum lethal dose, but no more than 1 M.L.D. was influenced with C 40, C 110, and optochin, while with C 29 and C 36, death of the mice ensued on less than 1 M.L.D.

The rapidity with which the bactericidal activity of these compounds is neutralized within the animal body is shown in Table XIII.

A therapeutic dose of each drug was injected into the peritoneal cavity of mice in triplicate, and 5 minutes later, 1, 10, and 20 M.L.D. of pneumococci were injected by the same route. An amount of chemical which in simultaneous injections kills 500 to 8,000 M.L.D. is fixed, changed, exhausted, or somehow destroyed in 5 minutes, so that all the mice injected with 10 M.L.D. died, and none receiving 20 M.L.D. survived.

TABLE XI.

Per Os Therapy after Intraperitoneal Injection of 2 M.L.D. of the Organism.

Chemical.	Single doses immediately after injection of organism.			Three doses, 2 hrs. apart; first immediately after injection of organism.		
	Dose.	No. of mice.	Length of life.	Dose.	No. of mice.	Length of life.
	mg.			*mg.*		
C 29	2.5	4	48 hrs.; 72 hrs.; 72 hrs.; 120 hrs.	2.5	3	24 hrs.; 24 hrs.; 48 hrs.
	1.25	3	48 " 48 " 96 "	1.25	3	24 " 24 " L. "
	0.625	3	72 " 48 " 48 "	0.625	3	48 " 72 " "
				0.25	3	48 " L.; L.
C 36	5.0	4	48 hrs.; L.; L.; L.	5.0	3	24 " 24 hrs.; 36 hrs.
	2.5	3	72 " " "	2.5	3	24 " 48 " L.
	1.25	3	72 " " "	1.25	3	48 " L.; L.
				0.625	3	54 " " "
C 40	5.0	4	48 hrs.; 48 hrs.; 48 hrs.; 54 hrs.	5.0	3	24 " 48 hrs. 48 hrs.
	2.5	3	L.; L.; L.	2.5	3	24 " 48 " 48 "
	1.25	3	72 hrs.; L.; L	1.25	3	48 " 48 " L.
				0.625	3	L.; L.; L.
C 110	5.0	4	48 hrs. 60 hrs.; 72 hrs.; 72 hrs.	5.0	3	24 hrs.; 24 hrs.; 48 hrs.
	2.5	3	48 " 96 " 72 "	2.5	3	24 " 48 " 60 "
	1.25	3	48 " 72 " 72 "	1.25	3	48 " L.; L.
				0.625	3	72 " " "
Optochin.	5.0	4	72 hrs.; L.; L.; L.	5.0	3	48 hrs.; 48 hrs.; 48 hrs.
	2.5	3	72 " 72 hrs.; 72 hrs.	2.5	3	48 " 72 " L.
	1.25	3	48 " 72 " 72 "	1.25	3	48 " L.; L.
				0.625	3	48 hrs.; L.

TABLE XII.

Per Os Therapy. Three Doses Repeated at 2 Hour Intervals; First Dose Simultaneous with Injection of the Organism.

Chemical	Dose. mg.	No. of mice.	Length of life. No. of M.L.D. injected intravenously.		Dose. mg.	No. of mice.	Length of life. No. of M.L.D. injected intraperitoneally.		
			8	1			2	20	40
C 29	0.25	4	24 hrs.; 24 hrs.; 48 " 6 days.	24 hrs.; 48 hrs.; 48 " 72 "	0.25	4	48 hrs.; 48 hrs.; L.; L.	96 hrs.; L.; L.; L.	48 hrs.; 72 hrs.; 72 " 72 "
C 36	0.5	4	36 " 36 hrs.; 4 days; 6 days.	36 " 48 " 96 " 6 days.	0.5	4	48 hrs.; 72 hrs.; L.; L.	48 hrs.; 48 hrs.; L.; L.	48 " 72 " 72 " L.
C 40	0.5	4	L.; L.; L.; L.	36 " 48 hrs. L.; L.	0.5	4	L.; L.; L.; L.	72 hrs.; L.; L.; L.	48 " 72 hrs.; 6 days; L.
C 110	1.0	4	" " " "	L.; L.; L.; L.	1.0	4	48 hrs.; L.; L.; L.	48 hrs.; L.; L.; L.	48 hrs.; 48 hrs.; L.; L.
Optochin.	1.0	4	" " " "	" " " "	1.0	4	48 hrs.; L.; L.; L.	48 hrs.; L.; L.; L.	48 hrs.; 48 hrs.; 48 " L.
Controls.	0	4	0.0005 cc. 6 days; L. L.; L.	0.0001 cc. 35 hrs.; 48 hrs.; 96 hrs.; 120 hrs.	0	4	48 hrs.; 48 hrs.; 48 " 48 "	36 hrs.; 36 hrs.; 36 " 36 "	36 hrs.; 36 hrs.; 36 " 36 "

TABLE XIII.

Protection Experiment. Drug Injected 5 Minutes before the Organism.

Chemical	Dose.	No. of mice.	Length of life.		
			No. of M.L.D.		
			1	10	20
	mg.				
C 29	0.5	3	36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.
C 36	1.0	3	12 " 72 " 36 "	36 " 36 " 36 "	60 " 36 " 36 "
C 40	1.0	3	36 " L.; L.	36 " 48 " 240 "	36 " 36 " 72 "
C 110	1.0	3	36 " " " " "	36 " 72 " 120 "	36 " 72 " 72 "
Optochin.	2.0	3	48 " " " " "	36 " 72 " 72 "	36 " 60 " 72 "
Controls.	0	3	36 " 36 hrs.; 36 hrs.	36 " 36 " 36 "	36 " 36 " 36 "

Our work as so far reported has been restricted to a Type I pneumococcus (Neufeld). Moore[2] showed that there is a difference in the bactericidal action of optochin with pneumococci of the different types, but it is not necessarily specific for types. Using the same procedure of simultaneous intraperitoneal injection of organism and drug, described above in connection with the Neufeld pneumococcus, we have titrated an additional Type I and two each of Types II and III (Table XIV). It would appear from these results that the chemicals are most active against Type I, less so against Type II, and least against Type III. That this specificity perhaps is not as

<div align="center">TABLE XIV.</div>

Comparison of the Relative Protection Afforded by Different Drugs against Multiple Lethal Doses of Type I, II, and III Pneumococci. Simultaneous Injection Intraperitoneally of Drug and Organism.

Chemical.	No. of M.L.D.					
	Type I.		Type II.		Type III.	
	Neufeld.	No. 15.	No. 28.	No. 32.	No. 14.	No. 27.
C 29	500	100	400	20	0	1
C 36	5,000	1,000	400	50	0	0
C 40	8,000	1,000	500	100	0	1
C 110	6,000	1,000	400	50	10	1
Optochin.	700	400	400	50	10	1
No of organisms per M.L.D.	4	18	9	8	700,000	5,000,000

marked as it would seem is borne out, even in the case of these six strains, by two considerations. First, the virulence of the Type III strains was not as great as that of either of the Type I or of the Type II, the Type III having a minimum lethal dose in one strain of 700,000 and in the other of 5,000,000 organisms. It is possible that we are dealing not with type specificity but with organisms more or less resistant to chemicals and of varying numerical degrees of virulence. This is emphasized in the experiment shown in Table XV. Then, during another investigation, the virulence of the Type I (Neufeld) was reduced, so that 1 M.L.D. contained 7,000 instead of two to four organisms. This organism was used in multiple lethal doses, 1, 10, 100, and

TABLE XV.

Effect of Drugs on a Neufeld Type I Pneumococcus of Lowered Virulence (Decreased Approximately 4,000 Times).

Chemical.	Dose.	No of mice.	Length of life.			
			No. of M.L.D.			
			1,000	100	10	1
			No. of organisms.			
			6,500,000	650,000	65,000	7,000
	mg.					
C 29	0.5	2	48 hrs.; 48 hrs	48 hrs.; 48 hrs.	96 hrs.; 112 hrs	L.; L.
C 36	2.0	2	48 " 48 "	48 " 48 "	48 " 96 "	" "
C 40	1.0	2	48 " 48 "	48 " 48 "	L.; L.	" "
C 110	2.0	2	48 " 48 "	48 " 48 "	48 hrs.; L	" "
Optochin.	2.0	2	48 " 48 "	48 " 48 "	48 " 48 hrs	48 hrs.; 96 hrs.
Controls.	0	2	48 " 48 "	48 " 48 "	48 " · 48 "	48 " 48 "

1,000, and followed immediately by the chemical (simultaneous method). The results with organism in this stage of activity were very similar to those with Type III above, the chemicals, except C 40, being active against not more than 1 M.L.D.

Rabbit Experiment.

Two experiments were carried out with rabbits (Table XVI). Organisms and drugs were injected simultaneously. Regardless of toxicity, 25 mg. per kilo of each chemical were used. The pneumococcus (Neufeld) in a preliminary titration demonstrated a virulence such that 0.000001 cc. of a 6 hour serum broth culture

TABLE XVI.

Rabbit Experiment.

Chemical.	Pneumococci 0.00001 cc.					Pneumococci 0.000001 cc.				
	Dose per kilo.	Rabbit No.	Weight.	Life.	Culture.	Dose per kilo.	Rabbit No.	Weight.	Life.	Culture.
	mg.		*gm.*	*days*		*mg.*		*gm.*	*days*	
C 29	25	1	2,300	3	++	25	9	2,100	4	++
C 36	25	2	1,900	11	++	25	10	1,775	L.	
C 40	25	3	1,540	L.		25	11	2,000	"	
C 110	25	4	1,850	7	++	25	12	2,020	"	
Optochin.	25	5	1,950	2	++	25	13	1,675	3	++
	25	6	2,000	3	++	25	14	2,200	3	++
Controls.	0	7	1,800	3	++	0	15	1,950	3	++
	0	8	2,100	3	++	0	16	2,100	3	++

killed a 2 kilo rabbit in 3 days. In the first experiment with 0.0001 cc., or 100 M.L.D., the rabbit treated with C 40 remained alive (30 days), those treated with C 36 and C 110 showed a slight delay, while the controls, the C 29, and the optochin animals succumbed in 2 or 3 days. In the second experiment the same procedure was carried out, except that 1 cc. of 0.00001 dilution of organisms was employed for injection. The C 29, optochin, and control animals died in 4, 3, and 3 days respectively, those of C 36, C 40, and C 110 survived.

These results show that the bactericidal activity of the aromatic compounds is not restricted to mice. Optochin seemingly does not act with sufficient rapidity in rabbits to kill this number of organisms *in vivo.* The results of this experiment confirm the work with optochin of Moore,[17] Scott,[18] and Lewis.[19]

[18] Scott, W. M., *J. Path. and Bact.*, 1914–15, xix, 130.

[19] Lewis, J. H., *Arch. Int. Med.*, 1918, xxii, 593.

DISCUSSION.

The aromatic compounds described are a series of chemicals possessing rapid bactericidal action both *in vitro* and *in vivo*. There is, however, a pronounced variation in their action from the bacteriotropic and organotropic standpoint. C 29 ranks first in rapidity of action *in vitro*, but because of its polytropic characteristics is the poorest *in vivo*. The para-hydroxy substitution product (C 36) is an improvement in that the decrease in bactericidal action is more than compensated for by the lessening of its toxicity for mice. The bactericidal activity *in vitro* of the meta-hydroxy compound (C 40) is almost as great as the unsubstituted benzene derivative (C 29), and its toxicity is reduced 50 per cent. The dihydroxybenzene compound (C 110), judged by its ability to kill multiple lethal doses of pneumococci *in vivo*, holds an intermediate position between the para and meta derivatives. Its *in vitro* bacteriotropic activity, however, is much less than that of both the other aromatic chemicals and optochin. This substance is an example of a drug which, when injected simultaneously with multiple lethal doses of a virulent pneumococcus into the peritoneal cavity of mice, causes the death of a greater number of organisms than a corresponding amount *in vitro*. Apparently in a therapeutic dose its activity is auxiliary or perhaps stimulatory to the other natural forces of an animal which combat an infection. From the above it may be seen that there is a relationship between chemical constitution and chemotherapeutic activity.

The ultimate test of a chemotherapeutic agent is whether its use results in the cure of an established infection. Mice are unsatisfactory animals with which to make this test. The incubation period for the pneumococcus is so short, as well as the length of life following a fatal dose of the organism, that any remedial measure, if treatment be delayed till the infection is systemic in nature, may give misleading results.

Our method of investigation has shown that when pneumococci and drugs are injected simultaneously into the peritoneal cavity the death of multiple lethal doses of the organisms results. Any delay in the administration of the drugs neutralizes their effectiveness. Although an experimental infection was arrested when treatment was

postponed 2 hours after the injection of multiple lethal doses of organism (with chemicals and pneumococci injected intraperitoneally), at no time have we been able to cure a mouse having a systemic infection. The treatment involving the longest delay was administered after an 8 hour interval. In this experiment, not reported above, mice were injected with 1 M.L.D. and treatment was begun 8 hours later. 60 per cent of the mice treated with the aromatic compounds, with the exception of C 29, survived. Inasmuch, however, as pneumococci did not appear in the blood stream of the controls until 12 hours had elapsed, this study merely proves the chemicals to have a local therapeutic action, though of a high degree of efficiency. However, that there is some therapeutic activity resulting from diffusion of the drugs, as tested by injecting chemicals and organisms through different routes, is shown by the favorable results obtained in *per os* therapy.

Binz,[20] in an extensive series of experiments, has shown that, along with other physiological and pharmacological attributes of certain of the cinchona alkaloids, (1) they are general protoplasmic poisons, (2) stimulating in small amounts the activity of ameba, Infusoria, and white blood cells, in larger amounts paralyzing, and (3) decreasing the oxidizing and reducing power of the blood and weakening the enzymotic action. The summary o. his work indicates that quinine in small amounts stimulates normal functional activity, in large amounts causing paralysis of this same mechanism. We are unable to say that these aromatic derivatives produce the same effect, but it has been shown that in experiments with pneumococcus infection of mice their action is zonal; that is, within certain dosage the bacteriotropic action of the chemicals is greater than the deleterious influence on the defensive mechanism of the animal. Amounts above this therapeutic dose, although non-lethal, paralyze processes vital for the animal's defense, permitting the invading organism to develop without restraint. It is difficult to understand why this large, non-lethal dose does not kill the bacteria, when seemingly more than a sufficient quantity to do so is injected. There is an apparent reversal of relationship from bacteriotropism with small doses to organotropism with the large. In other words, there is a greater affinity for the animal

[20] Binz, C., *Virchows Arch. path. Anat.* 1869, xlvi, 67; Das Chinin. Nach den neuern pharmakologischen Arbeiten dargestellt, Berlin, 1875.

tissue than for the pneumococci, when large amounts of chemical are injected. This zonal phenomenon was found to occur in intraperitoneal, subcutaneous, and *per os* administration of all the drugs, optochin having a less pronounced zonal effect than the aromatic compounds.

The question arises as to whether it would be advisable to use drugs for chemotherapeutic purpose that present this zonal phenomenon, which at first glance seems so discouraging. That this may not be an insuperable barrier is suggested by the fact that it is present in some degree in all drugs used in clinical medicine. Drugs in general have a therapeutic zone beyond which treatment cannot be carried with safety to the patient. The practical value of any therapeutic agent lies in its ability to produce desired effects in relatively non-toxic quantities.

The question naturally arises whether the aromatic cinchona compounds which are reported in this paper may have therapeutic applications in man. This cannot be answered until further and wider studies have been made. Our experiments have been confined almost entirely to the one animal species; but that other larger animals can be used may be inferred from the experiments successfully carried out on rabbits. That the list of synthesized pharmacological agents effective against a bacterial infection has been enlarged is obvious. But we feel that the time for the employment of any of the drugs here described in man has not yet arrived. Further investigations along the lines followed may not impossibly lead to the goal being sought.

CONCLUSION.

It has been shown with one strain of pneumococcus (Type I, Neufeld), that hydroquinine chloroacetanilide (C 29), hydroquinine *p*-chloroacetylaminophenol hydrochloride (C 36), hydroquinine *m*-chloroacetylaminophenol hydrochloride (C 40), and hyroquinine 4-chloroacetylaminopyrocathecol hydrochloride (C 110) have a rapid pneumococcidal activity both *in vitro* and in the peritoneal cavity of mice, and to a lesser extent in rabbits. In comparison, optochin is slower in action, but its power is not so easily destroyed either *in vitro* or *in vivo*.

The introduction of the hydroxy group of the benzene nucleus of hydroquinine chloroacetanilide changes the relationship between

organotropism and bacteriotropism. In comparing the rapidity of *in vitro* bactericidal action and intraperitoneal toxicity, C 29 exhibits the most rapid pneumococcidal action and is the most toxic for mice. C 36, the para-hydroxy derivative, is one-fifth as toxic as C 29 and only one-tenth less active bactericidally. C 40 is one-half as toxic and has approximately the same bactericidal power, while C 110 is one-eigth as toxic and one-fifth as pneumococcidal; and optochin is one-sixth as toxic and has one-fifth the bactericidal action. Arranged in the order of their ability to kill pneumococci when injected simultaneously with them into the peritoneal cavity, the drugs are: C 40, C 110, C 36, optochin, and C 29.

The chemotherapeutic action of the aromatic compounds is essentially local in character. But by *per os* therapy there is demonstrated a certain amount of diffusion of this activity, not shown by any other method of administration, C 40 and C 110 having about the same value as optochin.

Intravenous injection of the drugs in small doses destroyed to a greater or less extent the natural defenses of the animal, optochin being perhaps less injurious than the aromatic compounds. This same destruction of natural resistance followed intraperitoneal and subcutaneous injections of the chemicals as measured by intravenous injections of the organisms.

The maximum tolerant dose in a single injection (intraperitoneal) is not so efficacious as the same dose divided in fifths and injected at hour intervals. Optochin under these conditions is not so active as the aromatic compounds. In general, repeated doses are more curative than single.

There is a zone between the therapeutic and toxic doses, both single and repeated, for all these chemicals alike, where the natural resistance of the animal to an infection is reduced. This effect is noted especially with C 29, C 36, and C 40. In the case of optochin the therapeutic dose is nearer the toxic than with C 110, C 36, and C 40. Apparently these chemicals exhibit a variability in *in vivo* bactericidal activity according to different strains of pneumococci and numerical virulence.

We wish to thank Dr. W. A. Jacobs and Dr. M. Heidelberger for the supply of chemicals and other assistance.

STUDIES ON X-RAY EFFECTS.

X. The Biological Action of Small Doses of Low Frequency X-Rays.

By WARO NAKAHARA, Ph.D., and JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 32 and 33.

Attempts have been made in this laboratory to compare the biological action of the soft or low frequency and the hard or high frequency x-rays, but the impossibility of comparing doses of the two types of rays with our present apparatus and measuring devices has rendered the results of these studies of little value. However, by varying the doses of the soft and hard rays there is one point which seems definite; namely, that with the softer rays it is possible to induce an apparent stimulation of the lymphoid cells which is preceded by only a very short and transitory period of depression.[1–4] With the harder rays the stimulation phase is less pronounced, if present at all in the dosages employed by us. Russ, Chambers, Scott, and Mottram[5] have succeeded in bringing about an increase in the circulating lymphocytes of rats by repeated small exposures to x-rays described by them as being of "medium" or "medium soft" quality. Our first satisfactory results were obtained with the tube operated at a spark-gap of about $1\frac{1}{2}$ inches. Later experiments with a spark-gap of about $\frac{3}{4}$ inch gave a more uniform and pronounced reaction.

The present study has to do with the effect of still softer x-rays than those used in previous experiments. For the generation of these

[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

[2] Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

[3] Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

[4] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[5] Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

rays a special water-cooled tube has been devised with a window of thin glass which will allow the passage of rays usually held back by the thicker glass of the standard tubes.[6]

Production of Lymphocytosis.

The following experiments were planned to determine the time of exposure to the soft rays necessary for the production of a maximum stimulation of the lymphoid system of mice.

Experiment 1.—Forty-five white mice were divided into nine groups of five mice each. The groups were exposed to x-rays for $\frac{1}{2}$, 1, 2$\frac{1}{2}$, 4, 5, 10, 20, 30, or 60 minutes respectively. The special tube described above was used with $\frac{1}{8}$ inch spark-gap, 11 milliamperes, and 6 inches distance, for the treatment. A cardboard was placed over the animal container for protection against the heat. Blood counts were made on all of the animals before and 1 week after the treatment. The mice given the 1 minute exposure showed a definite lymphocytosis, but no consistent change was noted in the blood picture of the other groups.

Experiment 2.—Blood counts were made on thirty normal white mice and they were then exposed to x-rays given by the special tube with the governing factors the same as in Experiment 1, the length of the exposure being 1 minute. Counts were made at intervals afterwards. The changes in the lymphocytes are shown in Text-fig. 1 by a composite curve and the results for the individual animals in Table I.

The counts showed that there was no definite alteration in the absolute number of polymorphonuclear leucocytes, but the lymphocytes were increased to a level considerably above that of the initial count. By the 2nd day these latter cells had almost doubled their number and continued to increase with some variations until the 14th day, after which they subsided but were still above their initial level on the 18th day. There was considerable variation in the extent and duration of the reaction in individual mice, as shown in Table I and in Text-fig. 2. In only two animals was there a drop in the lymphocytes at the time of the first count after the x-ray treatment. One of these, however, showed a reaction later with an increase of over 30 per cent, but in the other no stimulation was observed. It should be noted here that none of these counts were made soon enough after the

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[6] We wish to acknowledge our indebtedness to the Research Laboratory of the General Electric Company for the design and construction of this tube.

x-ray treatment to demonstrate the amount of depression preceding the stimulation, for this point has been adequately dealt with in previous publications.[7] The increase in number of circulating lymphocytes after this dose of x-rays is of greater magnitude and more constant than that previously induced by this agent.
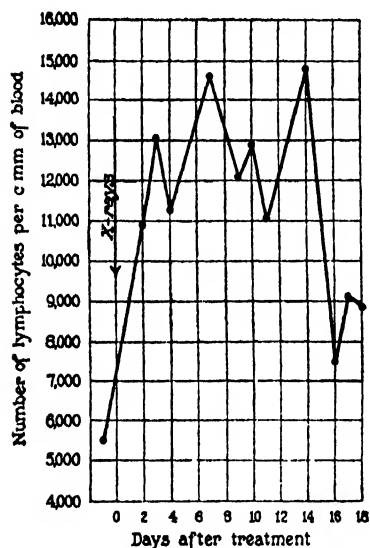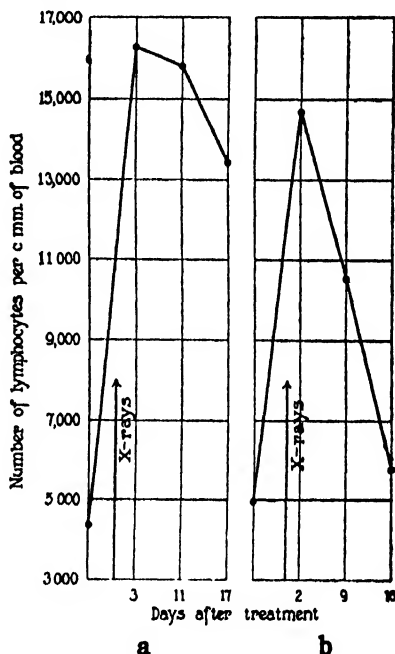
TEXT-FIG. 1.   TEXT-FIG. 2.

TEXT-FIG. 1. Composite curve of lymphocyte counts on thirty-five mice before and after an exposure to low frequency x-rays.

TEXT-FIG. 2, a and b. (a) Curve of lymphocyte counts on Mouse 23 in Table I. (b) Curve of lymphocyte counts on Mouse 12 in the same table.

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[7] In a recent article Mottram and Russ (Mottram, J. C., and Russ, S., *J. Exp. Med.*, 1921, xxxiv, 271) have objected to our use of the term "stimulating" doses of x-rays in describing the effect of this agent on the lymphoid tissue. They object to this term on the basis of the fact that the stimulation is preceded by a short period of depression. This point was noted in our first publication on the subject[1] and has been repeatedly referred to in subsequent articles.[4,9] The fact that with a suitable small dose of x-rays a stimulation of the lymphoid tissue is produced with only a slight and transitory depression, while with larger doses a more marked and lasting depression is induced with no subsequent stimulation, seems to us to justify the use of the expression.

TABLE I.

Mouse No.	Count before treatment.	Count after treatment. No. of days after x-ray treatment.										
		2	3	4	7	9	10	11	14	16	17	18
1	6,018				15,908				13,250			8,430
2	6,541				11,105				17,713			
3	5,897				14,455							
4	5,899				16,210				14,446			
5	5,526				15,463				14,744			
6	5,823	11,041				12,252				7,091		
7	4,617	7,658										
8	4,336	12,070				16,532				11,645		
9	6,585	17,070				10,603				8,455		
10	4,239	10,972				13,650				9,423		
11	4,288	7,604				11,766				5,471		
12	4,998	14,734				10,567				5,816		
13	7,104	7,070				12,786				7,005		
14	5,477	7,406				10,472				6,808		
15	5,185	12,878				10,332				6,520		
16	6,110		16,122				12,800				8,888	
17	6,113		12,775				16,861				7,183	
18	4,215		9,435				12,890				6,008	
19	4,909		12,002				7,338				5,360	
20	4,790		7,007				9,495				9,231	
21	3,714		12,064				9,304				8,232	
22	4,145		10,989				12,543				9,382	
23	4,425		16,261				15,814				13,209	

6,203		18,252				15,681				12,124	
7,155		15,352				17,055				12,836	
3,594			7,527				9,303				9,897
9,313			7,027				8,975				6,675
4,428			8,815				7,196				6,878
5,264			3,767				7,986				10,178
4,404			12,452				12,870				
3,974			15,300				15,082				8,571
8,330			13,167				10,277				14,796
7,329			16,480				17,274				9,893
6,175			16,787				12,894				5,092
5,635			11,851				8,518				
5,507	10,850	13,026	11,317	14,628	12,105	12,978	11,037	15,038	7,581	9,245	8,934

(Row labels, top to bottom: 24, 25, 26, 27*, 28, 29, 30, 31, 32, 33, 34, 35, Average.)

The counts before treatment were made a number of days in advance so that no animal had more than one count in a week. For example, on the group of mice counted 2 days after treatment, the pretreatment count was made 5 days before, thus allowing a week between counts.

Histological Changes.

The material for this study was collected from twenty-six mice which were carried as a parallel series to the above experiment in which blood changes were observed.   After exposure to x-rays (special tube, spark-gap $\frac{1}{8}$ inch, milliamperes 11, distance 6 inches, time 1 minute) these animals were killed in groups at intervals of 24 hours, 4 days, 7 days, 10 days, and 14 days.   The spleen, lymph nodes, bone marrow, thymus, thyroid, liver, kidney, suprarenal, ovary, and testis were taken for examination.   Carnoy 6–3–1 solution was used for fixation and the sections were stained with Heidenhain's iron-hematoxylin for mitotic figures or with Ehrlich's acid hematoxylin and eosin.

Lymphoid Organs.—The changes in the spleen and lymph nodes were practically identical and will be described together.   24 hours after treatment there was no demonstrable change in the lymphoid organs.   At the 4 day period numerous mitotic figures were found in all of these organs, a condition which our previous work indicates is associated with the increased production of lymphoid cells.   This condition was found equally as pronounced in the animals killed on the 7th day after treatment.   The organs from eleven mice were examined for these two periods and without exception this increase in proliferation was observed.   The animals killed on the 10th and 14th days after treatment showed that the proliferative activity had subsided to about the normal rate.

Suprarenal Glands.—The suprarenals were found at autopsy to be distinctly reddish in color and somewhat enlarged.   Microscopic examination showed the sinus-like spaces between the cortex and medulla to be much distended with blood, and this dilatation extended to the capillaries between the cortical cell columns, separating these columns by a wide margin (Figs. 1 and 2).   The sinusoids of the medulla showed little if any modification.   There was no sign of hemorrhage or of necrosis of the suprarenal tissue in any of the specimens examined.   This vascular change was observed in varying degrees in all of the mice examined except those killed 24 hours after the treatment.   In addition to this engorgement of the capillaries, a proportion of the animals killed between 7 and 14 days after the

x-ray treatment showed a pronounced perivascular infiltration in the cortex along the fibrous capsule and often extending down to the zona reticularis along the cortical cell columns.

Other Organs.—There was no histological evidence of changes in the other organs examined after the x-ray treatment. Even the germ cells of the fourteen ovaries and twelve testes examined, cells well known to be extremely sensitive, showed no damage which could be detected microscopically (Figs. 3 and 4). In a few instances the interstitial tissue of the testis was found to be hypertrophied. Distinct perivascular lymphoid infiltration was observed in a number of the livers and kidneys examined, but these findings were not uniform enough to be of great importance. This condition is also found occasionally in normal animals.

Effect on Resistance to Transplantable Cancer.

In previous publications it has been shown that a small dose of x-rays sufficient to stimulate the lymphocytes will increase the resistance of mice to transplanted cancer.[1,8,9,10] Russ, Chambers, Scott, and Mottram[6] have shown that the same condition holds for rats. As noted above, a more pronounced reaction of the lymphoid tissue is induced by the treatment outlined here than by the dosage previously used. It is of interest, therefore, to determine the effect of the very soft rays on the resistance to cancer.

Experiments 3 to 9.—Normal white mice of about the same age and size were exposed to a dose of x-rays governed by the same factors as those used in Experiment 2, with the special x-ray tube described above. These animals were inoculated in groups from 3 to 14 days after the treatment, with a graft of a transplantable tumor (Bashford Adenocarcinoma No. 63) along with a suitable number of controls. The results of seven such experiments are given in Table II.

From these experiments it is seen that no immunity is evident when the inoculation is made as early as 3 days after the x-ray treatment, a result which corresponds with the earlier experiments in which the standard Coolidge tube was used. Among 86 mice inoculated from 7

[8] Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35.

[9] Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

[10] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

TABLE II.

Experiment No.	Interval between x-ray exposure and tumor inoculation.	Immunity in x-rayed animals.	Immunity in control animals.
	days		
3	3	0.0 per cent (9 mice).	0.0 per cent (10 mice).
4	7	30.0 " " (10 ").	11.1 " " (9 ").
5	7	28.5 " " (17 ").	9.9 " " (11 ").
6	10	37.5 " " (8 ").	0.0 " " (10 ").
7	10	33.3 " " (18 ").	0.0 " " (10 ").
8	10	57.8 " " (19 ").	10.0 " " (10 ").
9	14	20.0 " " (10 ").	0.0 " " (7 ").
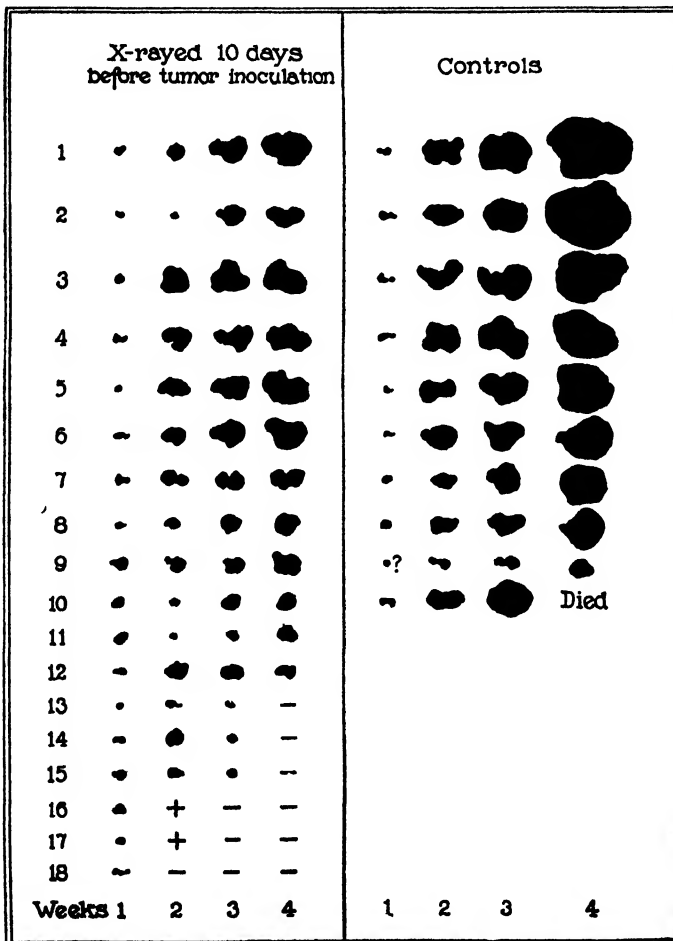
TEXT-FIG. 3. Experiment 7. The rate of growth of Bashford Adenocarcinoma No. 63 in mice given a small dose of low frequency x-rays 10 days before inoculation, contrasted with the rate of growth in untreated mice.

to 14 days after the treatment, 34.4 per cent were immune, while in 57 control mice inoculated with the same tumor there were only 5.1 per cent immune. The largest proportion of resistant mice was found among the animals treated 10 days after inoculation, with 42.8 per

X-rayed 10 days before tumor inoculation			Controls		
1	●	●	●	●	Died
2	●	●	●	●	Died
3	●	●	●	●	●
4	●	●	●	●	●
5	●	● Died	+	●	●
6	●	●	●	●	●
7	+	●	●	●	●
8	+	●	●	●	●
9	●	●	—	●	●
10	●	●	—	+	—	—
11	●	+	—		
12	+	—	—		
13	+	—	—		
14	+	+?	—		
15	+	+	—		
16	+	—	—		
17	+	—	—		
18	+	—	—		
19	+	—	—		
Weeks 1	2	3	1	2	3

TEXT-FIG. 4. Experiment 8. The rate of growth of Bashford Adenocarcinoma No. 63 in mice given a small dose of low frequency x-rays 10 days before inoculation, contrasted with the rate of growth in untreated mice.

cent in 45 mice against 3.3 per cent in 30 control mice. Text-figs. 3 and 4 show that the rate of growth of the tumor is slower in the treated animals than in the controls, and that in a proportion of the treated mice tumors after a period of growth are absorbed, a condition which rarely occurs with this tumor in normal mice.

The Absorption Coefficient of X-Rays Produced by a Voltage Measured by a ½ Inch Spark-Gap.[11]

It is of considerable interest in the light of the above experiments to arrive at some idea of the amount of penetration of the soft rays. The very soft rays produced by a voltage measured by a ½ inch point spark-gap were found to be practically homogeneous with a mass absorption coefficient equal to 3.4 for water. This value includes the mass-scattering coefficient usually taken as 0.2.

The value was measured photographically. A series of small areas of a single film was exposed to the rays for various times. A second series on the same film was exposed for various times to rays which had passed through layers of water of various thicknesses. After development the areas of the second series were matched for blackness against those of the first series and the relative intensities of the rays transmitted by the water thus discovered. A preliminary experiment gave the value for the absorption coefficient which was used to calculate the exposures through various depths of water necessary to give equal degrees of blackening. Another film was made accordingly and the exposed areas were found to be of uniform density. It will be noticed that, according to the quantum law, the wave-lengths produced by a ½ inch gap are in the neighborhood of the K characteristic absorption discontinuities of the silver and bromine of the plate. In spite of this it can be shown that the constant value found for the absorption coefficient is a proof of homogeneity.

The mass absorption coefficient of these rays in animal tissues may be assumed to be somewhat smaller than for water. A mouse skin weighing 0.2 gm. per cm. should stop less than one-half of the rays.

The relative amount penetrating various depths of water when this water-cooled tube is operated at ½ inch spark-gap is as follows:

Depth.	Intensity.
cm.	
0	1.0 (taken as standard).
¼	0.43
½	0.18
¾	0.076
1	0.032
1¼	0.0133
1½	0.0056

[11] We are indebted to Dr. Harry Clark for the measurements recorded.

DISCUSSION.

The comparison of the biological effect of the hard and soft rays is a matter of considerable interest at the present moment but no really satisfactory standard of measurement is available. Such comparisons, therefore, must await a further understanding of the complex nature of the biological changes and the underlying factors bringing about these changes.

As far as our present knowledge extends the only known physical or chemical change induced by x-rays depends on the power of this agent to ionize. If ionization be the underlying factor responsible for the biological changes, it is necessary to determine why some types of animal cells are so profoundly affected in the absence of demonstrable changes in other cells. However, the solution of such problems as this must await further development in biophysics.

In this communication evidence is presented of a biological change induced by a small dose of the very soft x-rays; namely, a stimulation of the lymphoid cells preceded in all probability by a small amount of destruction. We have not been able to induce a reaction of this nature with the harder rays given in varying dosage. Until it is determined that the dosage is the same it will not be possible to say whether this apparent difference in biological action of the soft and hard rays is a real one.

Considering the smallness of the dose, the fact that 57 per cent of the x-rays used here is absorbed in the first $\frac{1}{4}$ cm. of tissue and that only 3.2 per cent penetrated to the depth of 1 cm. strongly suggests that the changes in the lymphoid organs are not the result of the direct action of the x-rays. The fact also that the deeper nodes react as much as the more superficial ones strengthens this idea. The significance of the changes in the suprarenals can only be determined by further study. It is noteworthy that the testicle and ovary, organs supposed to be extremely sensitive, were unaffected by this treatment.

The virulence of the strain of tumor used to test the resistance of the mice was such that the so called natural resistance was almost completely obviated, a fact which renders the result of the inoculation of the treated mice the more striking.

SUMMARY.

A study has been made of the biological effect of a small dose of soft x-rays given off by a special water-cooled tube with a window of thin glass, operated at ½ inch spark-gap and 11 milliamperes.   Mice exposed for 1 minute show 2 days later in the blood an increase in the number of lymphocytes and in the lymphoid organs an increased number of mitotic figures.   There occurs also a marked dilatation of the vessels of the suprarenals, particularly between the cortex and medulla.   The latter condition did not appear until after 24 hours and was still present 14 days after the treatment.   No change was detected in other organs.

Mice treated in this way showed a high degree of resistance to cancer transplants.   The amount of resistance varied with the time of the inoculation after the treatment.   The resistance was not increased before 3 days after and was at its highest point 10 days after the treatment.

EXPLANATION OF PLATES.

PLATE 32.

FIG. 1. Suprarenal gland of mouse 7 days after an exposure to low frequency x-rays, showing dilatation and engorgement of capillaries.

FIG. 2. The same as Fig. 1; higher power view.

PLATE 33.

. FIG. 3. Testis of mouse 24 hours after an exposure to low frequency x-rays. Spermatocytes in the periphery of follicles in the so called syncopic stage.   In the large follicle to the left are seen several examples of the first spermatocytic division.

FIG. 4. Ovary of mouse 24 hours after an exposure to low frequency x-rays.
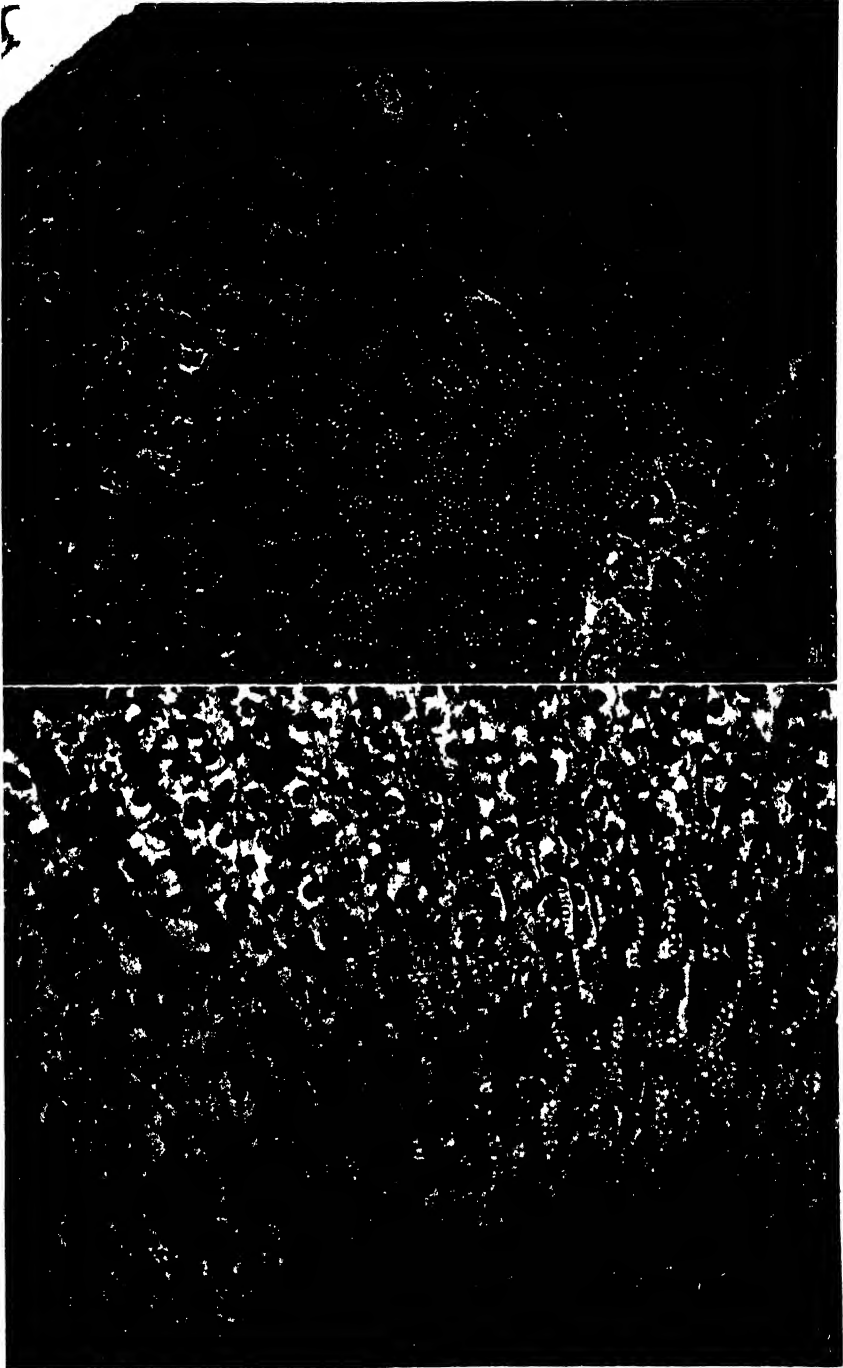
(Nakahara and Murphy: Studies on x-ray effects. X.)

STUDIES ON X-RAY EFFECTS.

XI. The Fate of Cancer Grafts Implanted in Subcutaneous Tissue Previously Exposed to X-Rays.

By J. HENG LIU, M.D., ERNEST STURM, and JAMES B. MURPHY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

It has been shown in this laboratory[1] that an erythema dose of x-rays produces in the skin layers of mice a reaction characterized by lymphoid infiltration coincident with a local increase in resistance to transplanted cancer. Moreover, it was found that while the x-rayed areas are refractory to subsequent intracutaneous inoculation of cancer, subcutaneous inoculations beneath the x-rayed areas result in the same number of growths as in the normal areas. These experiments were offered as a probable explanation of the fact that many skin cancers in man are readily influenced by x-rays while identical cancers in the superficial lymph nodes are controlled with great difficulty, if at all, by the treatment.

The exposure of open wounds to x-rays at the time of operation for the removal of cancer in man has been frequently advocated, particularly in cancer of the breast[2] for the purpose of destroying any remaining cancerous tissue. While the results are stated to have been satisfactory, it is difficult to judge the value of the method in the treatment of human cancer in which it is impossible to provide a suitable number of controls.

Hill, Morton, and Witherbee have shown that mouse cancer cells are not killed by x-rays *in vitro* in doses much greater than those tolerated by the skin.[3] Yet as stated above, a much smaller dose will render the skin resistant to implants of a strain of this same cancer.

[1] Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299.

[2] Pfahler, G. E., in Deaver and McFarland, The breast, its anomalies, its diseases and their treatment, Philadelphia, 1917, 651.

[3] Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

Hence it became desirable to determine whether or not direct exposure of the deeper tissues renders them refractory to implanted cancer.

Tumor Inoculation after Exposure of the Subcutaneous Tissue to X-Rays.

Normal white mice were etherized, shaved over the abdomen, and under aseptic conditions a rectangular skin flap was made beginning at the midline and extending about 1.5 cm. laterally across the abdomen. The incision was made so as to leave the skin attached at the distal end and the flap was separated from the underlying structures so as to include all of the subcutaneous tissue down to the muscle. The under side of the flap and the exposed muscle, after being covered with gauze wet with salt solution, were given directly a dose of x-rays governed by the following factors: 3 inch spark-gap, 10 milliamperes, 6 inch distance, 2½ minutes. With the exception of this area the animal's body was protected by sheet lead. Immediately after the treatment a cancer graft was introduced into the loose connective tissue of the under side of the flap and the skin sutured back into place. As a control, another series of animals was treated in precisely the same fashion except that no x-rays were given.

In practically all of the animals of both series the wounds healed within 5 or 6 days by primary intention, with no detectable difference between the x-rayed and control animals. Weekly examinations were made to determine the fate of the cancer grafts and later verified by autopsy.

The results of four such experiments are given in Table I.

TABLE I.

Experiment No.	Resistant x-rayed mice.	Resistant control mice.
1	66.6 per cent (15 mice).	23.0 per cent (13 mice).
2	68.4 " " (19 ").	0.0 " " (6 ").
3	73.3 " " (15 ").	29.4 " " (17 ").
4	50.0 " " (10 ").	0.0 " " (10 ").
Average...	66.1 per cent (59 mice).	17.4 per cent (46 mice).

It is apparent from these experiments that an erythema dose of x-rays given directly to the subcutaneous tissue brings about some change which renders this tissue decidedly less suitable as a soil for the growth of implanted cancer (Text-fig. 1). Another point of

Subcutaneous tissue x-rayed Tumor inoculated immediately after			Controls		
1 —	—	—	● ?	—	—
2 —	—	—	+ ?	—	—
3 —	—	—	— ?	—	—
4 —?	—	—	— ?	—	—
5 —	—	—	+ ?	—	—
6 +?	+?	—	●	●	●
7 —	—	—	●	●	●
8 —	—	—	+?	●	●
9 —	—	—	●	●	●
10 +	—	—	+?	●	●
11 —	—	—	●	●	●
12 —?	●	●	●	●	●
13 —	●	Died	—?	●	●
14 +?	●	●	●	●	●
15 —	●	●	●	●	●
16			●	●	●
17			●	●	●
Weeks 1	2	3	1	2	3

TEXT-FIG. 1. Results of inoculation of tumor into subcutaneous tissue previously exposed directly to x-rays, compared with a like inoculation in normal mice.

interest is that the cancer grafts which took in the x-rayed animals showed a tendency to grow inward toward the abdominal cavity with a flat inactive base on the side near the skin. Some of these did not produce even a slight elevation of the overlying skin and were only detected at autopsy.

To serve as a control for the above experiments and in order to confirm the finding that the effect of a local erythema dose of x-rays applied to the skin does not extend to the underlying subcutaneous tissue, the following experiment was carried out.

Subcutaneous Inoculation of Tumor after Exposure of the Skin to X-Rays.

Ten normal white mice were given a dose of x-rays over the left lower quadrant of the abdomen, the dose being governed by the same factors as those used in the preceding experiment. Immediately following the exposure, a skin flap was made in the x-rayed area and a cancer graft (Bashford No. 63) was introduced into the loose areolar tissue of the under side of the flap and the wound closed with sutures. The tumor grew in all of these animals, from which it may be concluded that an erythema dose of x-rays given to the intact skin does not increase the resistance of the underlying subcutaneous tissue.

Tumor Inoculation in a Protected Area after a Local Exposure of the Subcutaneous Tissue to X-Rays.

In order to determine whether the exposure to x-rays of a small area of subcutaneous tissue affects the general resistance to cancer grafts, a series of thirteen mice was operated upon and after the skin flap was made on the left side of the abdomen they were x-rayed over the open wound and then the flap was sutured back into place. Cancer grafts inoculated immediately afterwards in the right side resulted in tumors in 76.9 per cent of the animals, or in about the proportion observed in normal control mice.

Histological Changes after Direct Exposure of the Subcutaneous Tissue to X-Rays.

Two series of twelve mice each were shaved, and under ether flaps of skin and subcutaneous tissue were made over the left lower abdominal region. One series was x-rayed with an erythema dose directly on the under side of the skin flap and on the denuded surface of the abdominal muscle, the remaining parts of the animal being protected by sheet lead. The other series was operated on in the

same manner but not x-rayed. The wounds in both were sutured with great care as to the approximation of the skin edges. The animals were killed in groups of two, 24 hours, 3, 5, 7, 9, and 14 days after operation for examination.

Up to the 5th day the process of repair formed such a prominent part of the picture that it was impossible to detect any difference in the extent and character of the cellular infiltration from histological study. The 7 and 9 day preparations, however, in which the process of repair was in the last stages, showed distinctly that while in the animals not x-rayed only a layer of new connective tissue between the subcutaneous and muscle layers was slightly infiltrated with round cells, in the x-rayed animals large numbers of lymphocytes occurred, chiefly in the loose connective tissue, and in about half of the animals examined these cells had infiltrated the thickness of the muscle and formed a heavy layer between the muscle and the parietal peritoneum. At the end of 2 weeks this lymphocytic infiltration, although still evident, had subsided somewhat.

Two other groups of mice were operated on in the same manner as in the preceding experiment, and one of the groups was given a dose of x-rays over the exposed subcutaneous tissue and muscle. Before the skin flap was sutured back into place each animal received a cancer graft into the connective tissue underlying the flap. The microscopic appearances of the sections of tissue taken at intervals from the animals were so complicated, through operation, x-ray treatment, inoculation of tumor, natural differences in susceptibility, and in some cases, mild infections, that no conclusions in regard to the cellular reactions could be drawn.

DISCUSSION.

The observations reported in this paper bring out the fact that x-rays can be made to induce a local change in the subcutaneous tissue similar to that which this agent will induce in the skin. This change, in both instances, renders the locality resistant to the growth of implanted cancer cells, but does not affect the general resistance of the animal. This is an additional point to be taken into consideration in determining the method of treatment and the interpretation of clinical results following the use of x-rays as a therapeutic agent. The

clinician has rarely taken into account other possibilities than the direct destruction of the cancer cells.

X-rays under certain conditions materially increase the general resistance of the body to cancer, an observation made in this laboratory,[4] and later confirmed and extended by Russ, Mottram, and their coworkers.[5]   On the other hand, excessive doses of x-rays are capable of lowering both natural and induced resistance to cancer.[6]   The amount of this agent required to kill mouse cancer cells is many times that which can be tolerated by the skin, yet as shown above a mild erythema dose is sufficient to render the skin and under proper conditions the subcutaneous tissue antagonistic to the growth of implanted cancer.   It is undetermined which of these various qualities of x-rays are responsible for the successes and failures in the treatment of human cancer.   It would seem of prime importance to estimate the relative value of these effects, for it is not beyond the possible that a method of treatment could be devised which would make use of the favorable and eliminate the unfavorable action of this agent.

SUMMARY.

An erythema dose of x-rays given direct to the exposed subcutaneous tissue and muscle greatly diminishes the susceptibility of the exposed area to transplanted cancer.   The same dose given over the intact skin does not affect the resisting power of the underlying subcutaneous tissue.

Histological examination shows that a few days after the exposure of·the subcutaneous tissue there is a lymphoid infiltration of this tissue, which infiltration sometimes includes the muscle layers as well.

[4] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.   Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35.   Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.   Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

[5] Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.   Russ, S., Chambers, H., and Scott, G. M., *Proc. Roy. Soc. London, Series B*, 1921, xcii, 125.

[6] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.   Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.   Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917, xc, 1.

STUDIES ON LYMPHOID ACTIVITY.

VI. Immunity to Transplanted Cancer Induced by Injection of Olive Oil.

By WARO NAKAHARA, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 34 to 36.

It has been frequently suggested that the lymphoid cells are in some way concerned with the absorption and digestion of fats and lipoids. Recently a number of investigators have reported on the cellular changes following injections of these substances. Ramond[1] found that olive oil injected intraperitoneally is gradually absorbed by white cells of the lymphoid variety, and Clark[2] noted that the subcutaneous injection of olive oil exerts what he considers to be a chemotactic influence on the lymphatic endothelium and lymphocytes. Bergel[3] confirmed and extended these observations by finding that the cellular exudate after an intrapleural or intraperitoneal injection in animals of fatty oil or oil emulsion of lecithin is almost entirely made up of the lymphoid type of cell.

It is well known that the local reaction following an injection of homologous living tissue in mice consists mainly of a lymphoid cell outpouring[4] similar to that described above. Murphy and Nakahara[5] observed that this local reaction is accompanied by evidences of increased proliferative activity among the lymphoblastic cells of the spleen and lymph nodes. It may be stated in passing that mice thus injected with homologous tissue become highly resistant to

[1] Ramond, M. F., *Compt. rend. Soc. biol.*, 1904, lvi, pt. 2, 95.

[2] Clark, E. R., and Clark, E. L., *Am. J. Anat.*, 1917, xxi, 421.

[3] Bergel, S., *Berl. klin. Woch.*, 1919, lvi, 915; *Z. exp. Path. u. Therap.*, 1920, xxi, 216; *Ergebn. inn. Med.*, 1921, xx, 36.

[4] Da Fano, C., *Z. Immunitätsforsch.*, *Orig.*, 1910, v, 1.

[5] Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

transplanted cancer.[6] A like stimulation of the proliferative activity of the lymphoid cells may be induced by certain physical agents,[7] with resultant increased resistance to cancer transplants.[8]

In view of these observations it was regarded as of interest to determine whether or not the local reaction to oil is accompanied by a general lymphoid stimulation and, if so, the effect on the resistance to cancer inoculation in mice.

General Lymphoid Response to Injections of Olive Oil.

Commercial olive oil,[9] described as the first expression, was used in the following experiments. The injections were made intraperitoneally, followed by a histological study of the general condition of the lymphoid organs, with special attention to the number of mitotic figures present as this had been shown to be a fair index of the degree of stimulation.[10]

Experiment 1.—Twenty-five normal white mice of about the same age and size were divided into five groups of five mice each. The mice of Group A received an intraperitoneal injection of 0.1 cc. of the oil, Group B received 0.2 cc., Group C, 0.3 cc., Group D, 0.5 cc., and Group E, 0.7 cc. One animal from each group was killed for histological study 24 hours, 48 hours, 3 days, 4 days, and 5 days after the injection.

Group A.—Mice of this group received each 0.1 cc. of the oil. No unusual features were found in lymphoid organs of any of the mice, excepting one that was killed 4 days after the injection, and the germ centers of the lymphoid tissue of this mouse showed a marked increase of mitotic figures.

Group B.—Mice of this group received each 0.2 cc. of the oil. The mouse killed 24 hours after the injection showed no unusual condition, but four others killed at 48 hours to 5 days did show, particularly in germ centers (Fig. 1), a definite increase in mitosis.

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[6] Bashford, E. F., *Brit. Med. J.*, 1906, ii, 209.

[7] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[8] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

[9] It was estimated that this product contained about 30 per cent of cottonseed oil. This was determined by the density of the brown color following the addition of nitric acid, using as a standard known dilutions of the cottonseed oil.

[10] Nakahara, W., and Murphy, Jas. B., *Anat. Rec.*, 1921, xxii, 107.

Group C.—Mice of this group received each 0.3 cc. of oil. An appreciable but less marked increase in the number of mitotic figures was present 48 hours to 5 days after the injection.

Group D.—Mice of this group each received 0.5 cc. of oil. Judged by the number of mitotic figures, proliferative activity was retarded in the 24 and 48 hour specimens and about normal in the remaining animals.

Group E.—Mice of this group each received 0.7 cc. of the oil. In mice killed at the 24 and 48 hour periods, the lymphoid organs, especially the spleens, were much reduced in size. These small lymphoid organs showed an almost complete suppression of mitosis, reduction in the amount of lymphoid tissue, and, in the splenic pulp, vacuoles of various sizes. At later periods, while the size of lymphoid organs was still small, the rate of mitosis was approximately normal.

The preceding experiment indicates that the most pronounced reaction in the lymphoid organs followed the intraperitoneal injection of a dose of 0.2 cc. of the oil.

Experiment 2.—Twelve normal white mice were injected intraperitoneally with 0.2 cc. of olive oil as in Group B of the preceding experiment. The mice were killed in groups of three each at 48 hour, and 4, 7, and 10 day intervals.

Of the three mice killed 48 hours after the injection, one showed unmistakable increase in the number of mitotic figures in the lymphoid germ centers, another a less striking but distinct increase, and a third mouse no appreciable increase.

At 4 and 7 day periods, all the mice showed a greatly increased number of mitotic figures. In one mouse killed at the 4 day period the mitosis was especially exaggerated, and was present not only in, but outside the germ centers.

At the 10 day period, mitotic figures were still abundant in two mice and there were a few in the third.

As a control to the above experiment, a number of normal mice were injected intraperitoneally with 0.2 cc. of liquid petrolatum without leading to an increase of the mitosis.

Other Organs.—The livers of a few of the mice injected with oil had many intracellular vacuoles suggestive of fatty inclusions. Also occasionally there were found a marked dilatation of the capillaries and sinus-like spaces of the suprarenal gland and an increase in the number of mitotic figures in the cortical cells (lymphocytes) of the thymus.[*] These findings were of irregular occurrence and, therefore, should not be classed as typical changes induced by the oil injection. No special alterations were noted in the thyroid glands, kidneys, or bone marrow.

Cytology of the Peritoneal Exudate.—Smears were taken of peritoneal fluid at autopsy in Experiments 1 and 2. As already pointed out by Bergel,[3] all the smears showed numerous cells of lymphoid group, including typical large and small lymphocytes, so called transitional cells, plasma cells, large cells resembling macrophages, and true endothelial cells. Morphologically, there is a complete series of intergradations from the typical small lymphocyte to the large macrophage-like cell, suggesting that they all belong to a single biologic group, a point which has been emphasized by Bergel.[3] At an early stage (24 hours after the oil injection), there is a considerable number of polymorphonuclear cells, particularly neutrophils and eosinophils, in addition to lymphoid cells, but the number of these granular cells soon falls off. At the 48 hour period, the lymphoid reaction is apparently at its height, while at this time the polymorphonuclear reaction has about subsided (Fig. 2). The local reaction occurs regardless of the amount of oil injected and lasts as long as the oil remains in the peritoneal cavity. When 0.2 cc. of olive oil was injected the reaction tended to subside within 10 days, or hand in hand with the gradual absorption of the oil.

The above experiments indicate that lymphoid tissue as a whole responds definitely to an intraperitoneal injection of olive oil, which, if given in the optimum quantity, brings about a marked stimulation of the proliferative activity of this tissue. Studies on the peritoneal exudates, moreover, confirmed the results reported by Ramond,[1] Clark,[2] and Bergel,[3] regarding the local cellular manifestations about the injected fatty and lipoidal substances.

The relation between the local lymphoid reaction and the stimulation of the germ centers cannot be determined directly. However, in view of the lipolytic function of lymphoid cells,[11] it does not seem improbable that the local lymphoid response to the injected oil is an expression of the attempt of the body to dispose of the injected material. If so, it is conceivable that an optimum grade of activity on the part of lymphoid cells may lead to the general lymphoid stimulation.

[11] Bergel, S., *Münch. med. Woch.*, 1909, lvi, 64. Fiessinger, N., and Marie, P.-L., *Compt. rend. Soc. biol.*, 1909, lxvii, pt 2, 77.

Cancer Inoculation Experiments.

The production by injection of olive oil of lymphoid stimulation, essentially similar to the condition of induced potential immunity to transplanted cancers, suggested the possibility of rendering animals resistant by the same method. Experiments were accordingly undertaken to determine this point.

The dose of olive oil was given in a single intraperitoneal injection. Cancer inoculation was made subcutaneously in the left groin, and the rate of growth of tumors was charted thereafter at weekly intervals for 3 weeks. The strain of cancer used was Bashford Adenocarcinoma No. 63, and all the mice were young adults of white variety from the same stock.

The Degree of Resistance in Relation to the Amount of Olive Oil Injected.

Experiment 3.—In order to determine the optimum dose of olive oil, this substance was injected into mice in different quantities, ranging from 0.1 to 0.7 cc. Cancer inoculation was made in every case 10 days after the injection. The results are summarized in Table I.

TABLE I.

Experiment 3.

Amount of olive oil.	Treated mice.		Controls.	
	Resistance	No. of mice.	Resistance.	No. of mice.
cc.	*per cent*		*per cent*	
0.1	20.5	19	0.0	10
0.2	40.0	18	0.0	19
0.3	25.0	20	5.5	21
0.5	6.1	23	11.1	9
0.7	0.0	9	0.0	9

Since the number of mice in each group is small, slight differences in the percentage of resistance in the several groups should not be considered as significant. However, the fact that emerges is that by injecting 0.2 cc. of olive oil 10 days before giving a cancer inoculation, mice are rendered more resistant to the inoculated cancer than they

normally are.   This same point was brought out in three additional experiments, the results of which are shown in Table II.[12]

TABLE II.

Experiments 4 to 6.

Experiment No.	Mice injected with olive oil 10 days before cancer inoculation.		Controls.	
	Resistance.	No. of mice.	Resistance.	No. of mice.
	per cent		*per cent*	
4	43.7	16	11.1	9
5	52.6	19	10.0	10
6 (Text-fig. 1).	41.0	20	10.5	19

The Degree of Resistance in Relation to the Time of Cancer Inoculation.

Experiment 7.—In the preceding experiment the cancer inoculations were made 10 days after the oil injection.   In order to ascertain the period at which the maximum degree of resistance is manifested, the inoculations in the following experiment were made at various intervals after an injection of 0.2 cc. of the oil.   The results are shown in Table III.

TABLE III.

Experiment 7.

Intervals between oil injection and cancer inoculation.	Resistance.	No. of mice.
days	*per cent*	
5	20.0	10
10	44.4	9
15	30.0	10
25	10.0	10
Control.	0.0	12

In all the former types of induced resistance to transplanted cancer so far studied, there is a period following the treatment during which there is slight, if any, evidence of resistance.   This is not only true as

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[12] The amount of olive oil should be slightly changed according to the size of the mouse.   For a large mouse, weighing over 25 gm., as much as 0.3 cc. can be given.

regards the injection of homologous living tissue,[13] but also equally after exposure to intense dry heat and after small doses of x-rays.[14]

TEXT-FIG. 1. Experiment 6. The rate of growth of Bashford Adenocarcinoma No. 63 in mice injected with 0.2 cc. of olive oil 10 days before inoculation, contrasted with the rate of growth in untreated mice.

[13] Bashford, E. F., Murray, J. A., and Cramer, W., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 180. Woglom, W. H., *J. Exp. Med.*, 1912, xvi, 629.

[14] Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

TABLE IV.

Experiments 8 to 10.

Experiment No.	Resistance.		
	Group A.*	Group B.	Group C
8 (Text-(fig. 2).	44.4 per cent (9 mice).	0.0 per cent (9 mice).	0.0 per cent (12 mice).
9	30.0 " " (10 ").	10.0 " " (10 ").	0.0 " " (10 ").
10	50.0 " " (8 ").	10.0 " " (10 ").	12.5 " " (8 ").

* Group A was made up of mice injected with the oil 10 days before the cancer inoculation. Group B mice were injected with the oil immediately before the inoculation. Group C comprised the control mice receiving no oil and inoculated with the same tumor.

TEXT-FIG. 2. Experiment 8. Effect of 0.2 cc. of olive oil on the rate of growth of Bashford Adenocarcinoma No. 63, when administered 10 days and immediately before inoculation.

That a similar state arises after an injection of olive oil is shown by the above experiment. A more complete test of this point is given in Table IV.

Other Oils.—Several other oils of different chemical constitution have been tested; namely, cod liver oil, cocoanut oil, sperm oil, and liquid petrolatum (Nujol). Cancer inoculations made 10 days after injection of 0.2 cc. of these several oils induced no appreciable resistance. The tests do not, however, exclude the possibility of a suitable dosage of these oils inducing a result comparable to that given by olive oil.

Histological Changes Accompanying the Resistance Induced by Olive Oil.

The following experiments were carried out in order to supply material for a histological study of the nature of the reaction accompanying the resistant state induced by olive oil.

Experiment 11.—Ten normal white mice were given an intraperitoneal injection of 0.2 cc. of olive oil each. 10 days later they were inoculated with fragments of a Bashford Adenocarcinoma No. 63, subcutaneously in the left groin. The mice were then killed in pairs 24 hours, 48 hours, 3 days, 4 days, and 5 days after the inoculation and the grafts and the lymphoid organs were removed for histological study.

Local Cellular Infiltration.—The occurrence of a characteristic exudate around the cancer grafts in resistant animals has long been known.[15] This local reaction, in which the cells of lymphoid variety take a prominent part, subsides rapidly after the necrosis of the grafts has become complete. On this account the grafts in the present experiment were removed at early periods.

Specimens taken 24 and 48 hours after inoculation showed various types of wandering cells, especially polymorphonuclear leucocytes and fibroblasts, collecting in a great number around the graft. At the 3 day period, however, much of the polymorphonuclear reaction had subsided and there was a marked infiltration of lymphocytes, plasma cells, and fibroblasts (Figs. 3 and 4) closely resembling the local re-

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[15] Burgess, A. M., *J. Med. Research*, 1909, xxi, 575. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1912, xv, 270. Tyzzer, E. E., *J. Med. Research*, 1915, xxxii, 201.

action known to occur in cancer-resistant animals.   Cell infiltration similar to the latter but in varying amounts was encountered in all the specimens taken at 4 and 5 day periods.

Stimulation of Lymphoid Tissue.—Animals resistant to cancer inoculation tend to develop on inoculation lymphoid hyperplasia.[16,17]   Murphy and Nakahara[5,17] have shown that this phenomenon, which is characterized by a marked increase in the number of mitotic figures in germ centers of lymphoid tissue, occurs very soon after cancer grafting in potentially resistant animals.

Spleens and lymph nodes taken as early as 24 hours after cancer inoculation in the above experiment showed that the number of mitotic figures in lymphoid tissue was greater than is seen in normal animals. At the 48 hour and 3 day periods the reaction appears to reach its height and at the latter periods mitotic figures were found in great numbers in the germ centers of the spleen (Fig. 5) and lymph nodes, and often in considerable numbers even in the lymph cord of the node (Fig. 6).   It should be stated that one animal each of the 4 and 5 day period failed to show any increase of mitotic figures, an irregularity without significance.

Blood Lymphocytosis.—Murphy and his associates have shown that a marked increase in the number of circulating lymphocytes accompanies the state of resistance to transplanted cancer.[18]   In order to ascertain whether or not such a lymphoid crisis occurs after cancer inoculation in the mice treated with olive oil, white cell counts were made of a number of such mice.

Experiment 12 (Text-Fig. 3).—Nineteen normal white mice were injected intraperitoneally with 0.2 cc. of olive oil and cancer inoculation was made in all of the mice 10 days afterward.   Ten of the mice proved to be resistant and nine susceptible to the inoculation.

The average number of lymphocytes per c.mm. of blood in the resistant mice, 1 day before the oil injection, was about 4,800 and of polymorphonuclear leucocytes about 4,700.   3 days after cancer inoculation the lymphocytes increased

[16] Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–18, xc, 1.

[17] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 327.

[18] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.   Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 433.

to about 11,800, while the polymorphonuclear cells were only slightly increased, being about 5,600. 2 weeks after cancer inoculation the lymphocyte count was still high, being about 10,000, while the polymorphonuclear leucocytes had returned to the initial level (about 4,600).

In cancer-susceptible mice, 1 day before the oil injection, the average number of lymphocytes and polymorphonuclear leucocytes was about 5,500 and 5,400
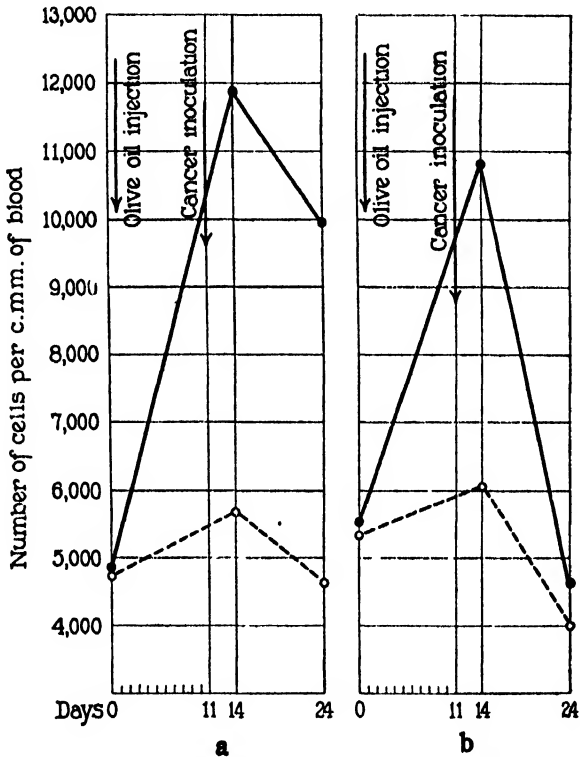
TEXT-FIG. 3. Experiment 12. Composite curves of white cell counts on mice injected with 0.2 cc. of olive oil and inoculated with Bashford Adenocarcinoma No. 63, 10 days later. (a) Composite curves from ten mice proved to be immune; (b) composite curves from nine mice proved to be susceptible. ———— Lymphocytes. .... Polymorphonuclears.

respectively. The counts were quite high 3 days after the inoculation, the lymphocytes being about 10,800 and the polymorphonuclear leucocytes about 6,000. At the end of the 2nd week after the cancer inoculation, all the cells were much reduced, the lymphocytes being about 4,600 and polymorphonuclear leucocytes about 4,000.

Experiment 13.—An experiment similar to the preceding one was carried out with ten mice, five of which were resistant and five susceptible.

In the resistant mice the average number of lymphocytes per c.mm. of blood 1 day before the oil injection was about 4,000; polymorphonuclear leucocytes about 4,400.   2 weeks after the cancer inoculation the lymphocytes were a little over 8,000 while the polymorphonuclear leucocytes had decreased to about 3,400.

In the susceptible mice, 1 day before the oil injection the lymphocytes were about 3,600 and the polymorphonuclear leucocytes, about 4,000.   2 weeks after cancer inoculation the lymphocytes were unchanged and the polymorphonuclear leucocytes had perceptibly increased (about 5,700).

The preceding experiments show that a characteristic lymphoid crisis occurs in the blood during the establishment of resistance to cancer grafting induced through olive oil injection.   Experiment 12 suggests that even in mice that proved to be susceptible there is apparently an inadequate reaction, which, however, is not of long duration.

It should also be stated that the injection of olive oil alone does not bring about a lymphocytosis.   White cell counts were made on ten normal mice injected with 0.2 cc. of olive oil.   In certain of the mice there was an increase and in others a decrease in number of circulating lymphocytes during the first 10 days after the oil injection, but the changes were too slight and too irregular to be of importance.

DISCUSSION.

The early work of Bashford and his coworkers[6] established the fact that resistance to transplanted cancer in mice could be induced by the inoculation of homologous living tissues.   Later Murphy and his associates[14] showed that resistance could be induced by the use of suitable doses of x-rays and intense dry heat.   The experiments reported in this paper demonstrate that resistance may be induced by still another means; namely, by the intraperitoneal injection of olive oil.   Thus it may be said that resistance to transplanted cancer can be induced by three classes of agents—homologous tissue, a biological agent; x-rays and heat, physical agents; and olive oil, a chemical agent.

There is little direct indication concerning the nature of the common factors responsible for the resistant state induced by these various agents but the manifestations associated with phenomena of resistance are the same regardless of the means used to induce this state.   These associated manifestations are, a latent period after the treatment, during which time there is no evidence of resistance,

a local cellular reaction about the inoculated cancer graft, an increase in the number of circulating lymphocytes, and a marked increase in the proliferative activity in the lymphoid organs. The indirect evidence associating the lymphoid cell with the mechanism of resistance to cancer is so strong as to leave little doubt that this cell has an important, if not the most important rôle in bringing about the resistant state.

SUMMARY.

The experiments reported in this paper show that it is possible to render mice resistant to transplanted cancer by injections of a suitable quantity of olive oil. In the course of the development of the resistance a definite period of latency is detectable following the oil injection, and the maximum degree of resistance appears at about the 10th day. This state of resistance, as has been determined by histological studies, is preceded by a proliferation of the cells of the lymphoid germ centers and, after the cancer inoculation, is associated with a lymphoid infiltration about the grafts, as well as by a second stimulation of the lymphoid germ centers and an increase in the number of the circulating lymphocytes.

EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Germ center of spleen 4 days after an intraperitoneal injection of 0.2 cc. of olive oil. M, mitotic figure.

FIG. 2. Peritoneal exudate 48 hours after an intraperitoneal injection of 0.2 cc. of olive oil.

PLATE 35.

FIG. 3. 48 hour old cancer grafts and surrounding connective tissue in mouse injected with 0.2 cc. of olive oil 10 days previous to cancer inoculation. Note the extensive cellular infiltration around the graft.

FIG. 4. High power view of an area of infiltration in the above specimen, showing the types of cells participating in the infiltration.

PLATE 36.

FIG. 5. Germ center of spleen of mouse injected with 0.2 cc. of olive oil and inoculated with cancer 10 days later. 3 days after cancer inoculation. M, mitotic figure.

FIG. 6. Medulla of lymph node of mouse treated similarly to that of Fig. 5. 3 days after cancer inoculation. M, mitotic figure.
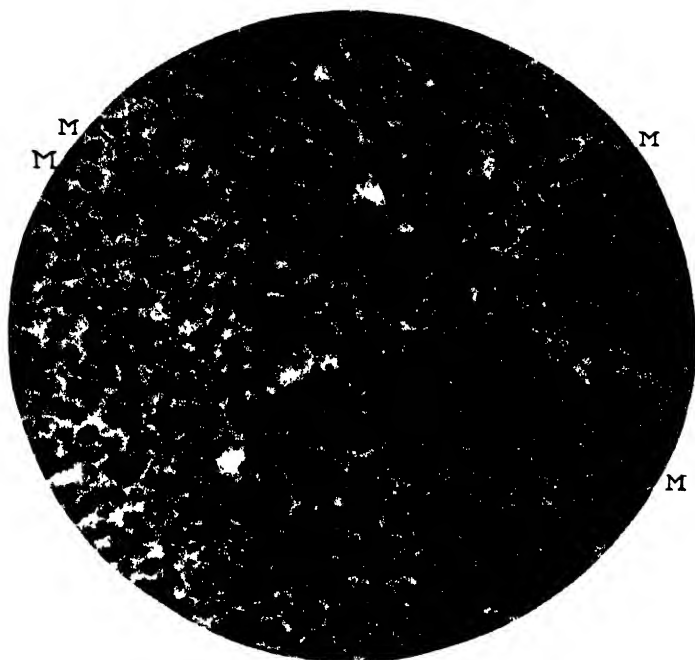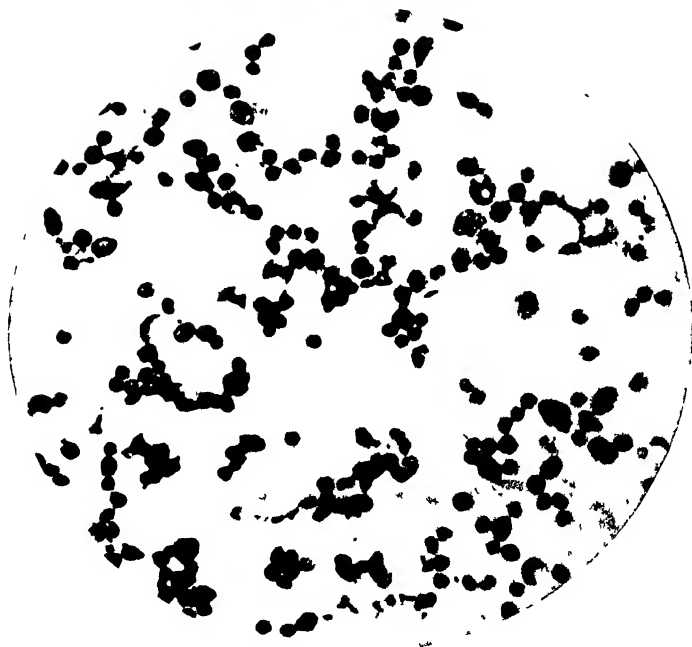
FIG 1.

FIG. 2.

(Nakahara: Lymphoid activity. VI.)
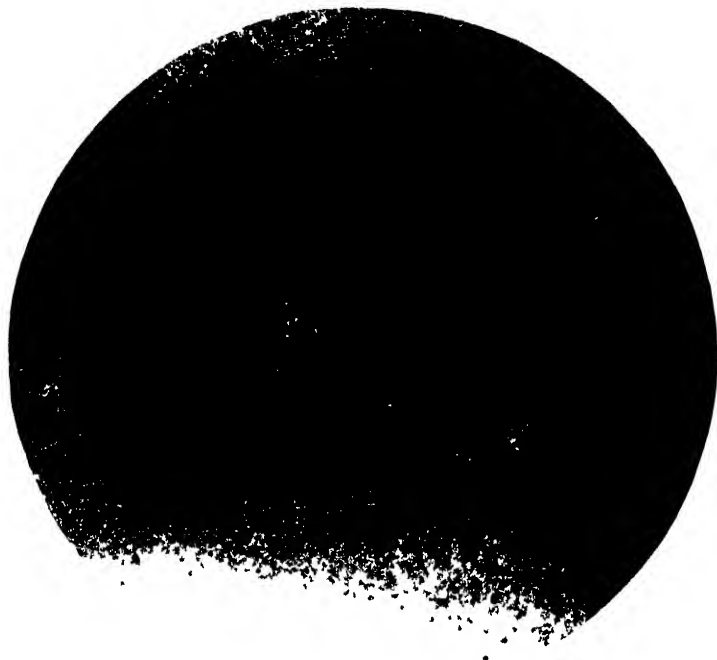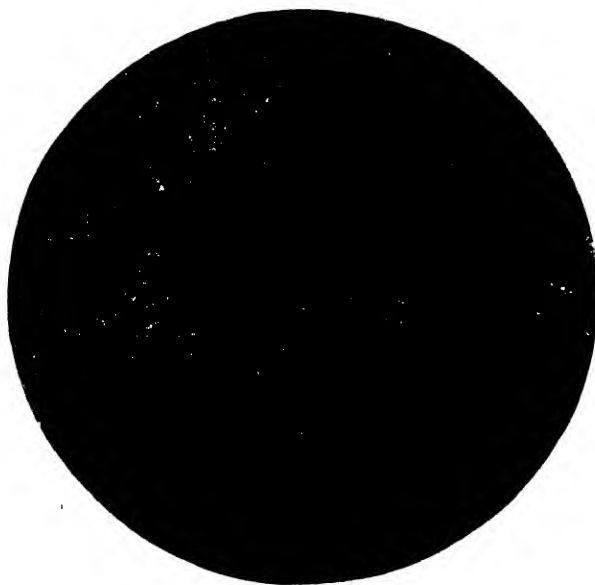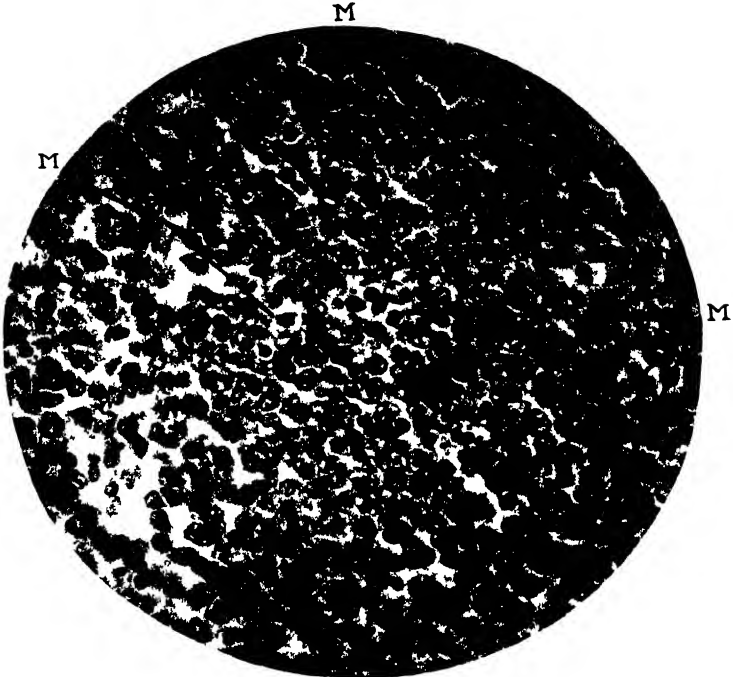
FIG. 3.

FIG 4

FIG 5

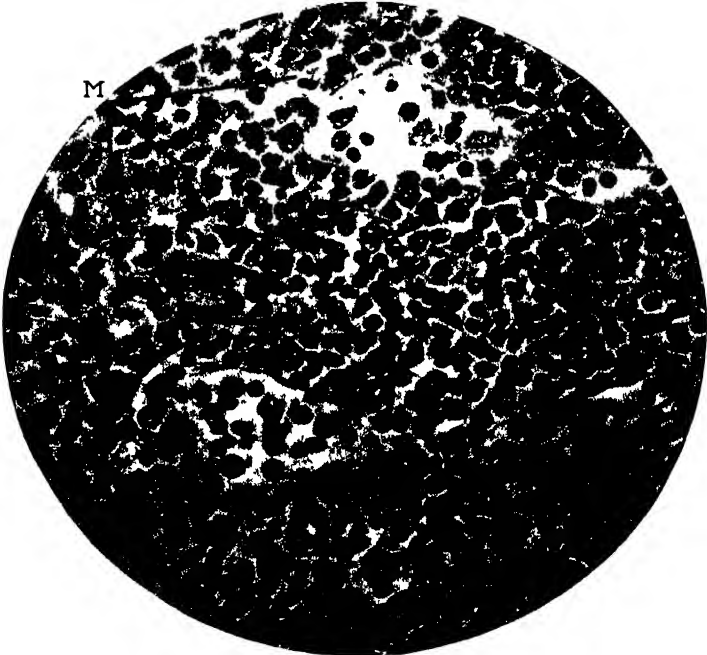

FIG 6

THE INFLUENCE OF X-RAYS ON THE PROPERTIES OF BLOOD.

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In the course of an experimental investigation concerning the nature of the reaction of the animal organism to x-rays we have made observations which we believe to be of sufficient interest to communicate in the form of a preliminary report. Our experiments were originally designed to throw some light on the question raised by the statement made by some roentgenologists that the characteristic illness frequently observed in individuals after an exposure to massive therapeutic doses of x-rays is due to a state of acidosis. The basis of this statement is apparently an empirical one since there is no rational evidence to support such an idea. We do not interpret the results of the experiments to be reported in this paper as a final answer to this question. We shall discuss this subject in a later paper.

Our procedure has been to study the numerical changes in the white cells of the blood, and the pH and bicarbonate content of the plasma in rabbits following an exposure of these animals to x-rays. Rabbits were chosen as the most convenient of the usual laboratory animals for our first observations on account of the facility with which they can be bled from the heart. We realize, however, that they are not ideal animals for our ultimate purpose. The plasma was obtained from blood drawn under paraffin oil into a tube which contained a sufficient amount of potassium oxalate to prevent coagulation. From 15 to 20 cc. of blood were drawn and the final oxalate concentration was about 0.3 per cent. Immediately after drawing the blood the plasma was separated from the cells by centrifuging. The surface of the oil was covered with low freezing point paraffin before centrifuging to prevent contact with air. We have found it possible to bleed a rabbit four or five times a week without any apparent effect on the

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general reaction of the animal, as regards the conditions of our investigation. For the pH determinations we have employed the colorimetric method developed by Cullen,[1] and for determining the bicarbonate content we have employed the procedure of Van Slyke.[2] The pH determinations have been checked electrometrically as have also the standard buffer solutions used in the colorimetric tests.

Preliminary observations showed the range of the pH value of rabbit plasma to be between 7.12 and 7.26, values which are lower than those stated by Hasselbalch and Lundsgaard (1912), 7.33 (electrometric), and Kurijama (1913), 7.4 to 7.5 (colorimetric). However, since the electrometric method employed in obtaining our values is more precise than was the method available at the time the values of Hasselbach and Lundsgaard were determined, we believe our values to be more nearly correct. We found the normal plasma bicarbonate content, expressed in volumes per cent of CO_2, to vary between 26 and 45. It is possible to obtain wide variations in a single rabbit unless careful attention is given to keeping the animal quiet during the process of bleeding. Feeding also plays a rôle in the variations of different samples of plasma but we have found it possible to control this factor by standardizing the amount of food and the time of feeding and the interval of time between the removal of animals from food before the beginning of the experiment.

The rabbits have been exposed to x-radiation from a Coolidge tube, the dosage factors being as follows: spark gap, 3 inches; current, 10 milliamperes; target distance, 6 inches; and time of exposure, 15 minutes. This is a massive dose of radiation of relatively long wave lengths, a large part of which are absorbed by the skin. This dose approaches the maximum amount that can be given without killing the animal and was purposely chosen as a starting point in order to obtain clear cut results. The exposures were made directly on the rabbit's abdomen which had been previously shaved.

EXPERIMENTAL.

1. Four rabbits were bled from the heart for plasma, then two of the animals were exposed to the radiation stated. The other two

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[1] Cullen, G. E., *J. Biol. Chem.*, 1922, 1, p. xvii.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

animals were not x-rayed. One control and one test animal were
bled again 24 hours later and the other control and test animals were
bled 48 hours after the x-ray exposure. 2 weeks later the test animals
were bled again. The observations made on the plasma obtained at
these times are given in Table I.

TABLE I.

Rabbit No.	Before radiation.		24 hrs. after radiation.		48 hrs. after radiation.		14 days after radiation.	
	pH	CO₂	pH	CO₂	pH	CO₂	pH	CO₂
		vol. per cent		*vol. per cent*		*vol. per cent*		*vol. per cent*
1 (x-rayed.)	7.16	33.2	7.34	51.0			7.22	51.2
3 "	7.21	35.6			7.35	49.6	7.23	48.6
2 (Normal.)	7.20	35.2	7.15	33.5				
4 "	7.19	38.6			7.14	34.7		

This experiment was repeated with results similar in magnitude to those given.

2. Two rabbits were bled then x-rayed as stated. Blood was
obtained from one of them an hour after the x-ray exposure, and from
the other 3 hours after. The results of the observations on the plasma
obtained are given in Table II.

TABLE II.

Before radiation.		1 hr. after radiation.		3 hrs. after radiation.	
pH	CO₂	pH	CO₂	pH	CO₂
	vol. per cent		*vol. per cent*		*vol. per cent*
7.26	38.5	7.30	48.0		
7.24	38.8			7.35	54.6

In other experiments observations have been made on the plasma
at various intervals following exposure to the x-rays and the above
results corroborated.

Blood counts have been made on rabbits which were bled as well
as x-rayed, and on others which were x-rayed only the changes do not
differ significantly. When counts are made at 15 minute intervals the
first decrease we have noted on the mononuclear elements occurs

$\frac{1}{2}$ hour after the x-ray exposure. After the first hour the interval between counts has been 1 hour and the maximum decrease occurs between 3 and 5 hours, the same period noted for the maximum change in the reaction of the plasma.

Summarized briefly the results of these experiments show that there exists a definite alkali excess in the body of the rabbit following an exposure to the dose of x-rays employed. Since there is an increase in the bicarbonate content of the plasma as well as an increase in the pH (*i.e.* a decrease in the C^{H+}), then evidently the $\frac{BA}{HA}$ ratio must be disturbed in a manner which defines a state of uncompensated alkali excess.[3] The maximum change in the reaction of the plasma is reached between 3 and 5 hours following exposure to radiation. The pH is observed to reach a normal level before the bicarbonate, which indicates the existence of a period of compensated alkali excess before the animal returns to normal. Evidence is found which significantly associates the point of maximum change in the reaction of the plasma with the maximum change noted in the white cell counts of the blood.

With these facts established we directed our attention to the possibility of producing a state of alkali excess in the rabbit by injecting sodium bicarbonate, and to the study of any chemical and morphological blood changes which might ensue. As a guide to the amount of salt to inject, we availed ourselves of the formula developed by Palmer and Van Slyke,[4] for bicarbonate administration with reference to the effect of a given amount of salt on the reaction of the body fluids. The formula is based on the fact that 1 gm. of $NaHCO_3$ yields 267 cc. of CO_2 measured under standard conditions. If now it is assumed that 0.7 of the body weight is fluid, it is obvious that there are 700 cc. of fluid for each kilo of body weight and 1 gm. of bicarbonate would increase the CO_2 content $\frac{267}{7W}$ volumes per cent. This expression simplifies to $\frac{38}{W}$ where W is the body weight expressed in kilograms. If now we let b = the volume per cent increase in CO_2, we

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlviii, 1.

[4] Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499

have the simple equation $b = \dfrac{38}{W}$. Obviously for g gm. of NaHCO$_3$

the equation becomes $g = \dfrac{bW}{38}$. As our results show, this equation

permits one to calculate with a good degree of accuracy the change to be expected.

Four rabbits were bled and the bicarbonate content and pH of their plasma was determined. Later they were given an intraperitoneal injection of sodium bicarbonate in amounts stated and the reaction of their plasma was again determined after an interval of 1 hour with the results given in Table III.

TABLE III.

Rabbit No.	Weight.	NaH CO$_3$ injected.	Before injection.		1 hr. after injection.			Difference between calculated and observed CO$_2$
			pH	CO$_2$	pH	CO$_2$ observed.	CO$_2$ calculated.	
	kilos	*gm.*		*vol. per cent*		*vol. per cent*	*vol. per cent*	*vol. per cent*
1	2.3	'	7.14	36.2	7.30	67.0	69.2	−2.2
2	2.3	3	7.19	35.6	7.42	88.1	85.2	+2.9
3	2.2	1	7.17	37.6	7.35	52.2	54.9	−2.7
4	2.1	1	7.22	40.6	7.36	55.4	59.9	−4.5

In other experiments similar observations have been made at varying intervals following injections of 1 gm. of bicarbonate. All injections were made into the peritoneal cavity. For periods after 1 hour the observed values are much less than the calculated values. The pH returns to normal in about 3 hours, but the bicarbonate content remains high up to 5 hours.

Observations on the white blood cells of these rabbits, as well as on other rabbits similarly treated show a diminution of white blood cells per cc. and the absolute counts show this to be due largely to the mononuclear forms. The maximum effect is noted 1 to 3 hours after the injection of the salt which time coincides with the period of maximum change in the reaction of the blood. This is true also in the rabbit following exposure to x-rays. Immediately following this maximum decrease there is a continued rise in the number of white blood cells per cc. and a rapid return to normal. It is possible, however, to maintain a low level of the mononuclear cells by repeated

injections of the salt. This is true in the guinea pig too, the only other animal to which we have so far extended these observations. Following an injection of 5 cc. of physiological saline solution we observe no significant change in the blood picture compared with blood counts on normal animals made at similar intervals.

DISCUSSION.

The facts thus presented develop an interesting analogy between the changes associated with x-ray effects and those found following injections of sodium bicarbonate. We do not feel justified at this time in attempting to make a definite interpretation of these results. The analogy, however, seems to be a striking one and it is very suggestive for further study, since it presents a possible opening for investigations concerning the mechanism of x-ray action on the animal body. Furthermore, evidence is found which justifies a doubt that the characteristic cell changes noted following x-ray exposures are due to the direct action of the x-rays. Additional force is given this doubt by the results of further experiments now in progress in which we are observing the effects accompanying the injection of other substances. So far we have used sodium chloride, sodium sulfate, monosodium phosphate, and glucose. Very definite changes are noted in the blood picture, but the data we have to date are not sufficient to permit of any further statement.

CONCLUSION.

When rabbits are exposed to x-radiation as described, there results a definite change in the $\frac{BA}{HA}$ ratio of their plasma. This change is evidently one which defines a state of uncompensated alkali excess.

The time required for a maximum change in the chemical reaction is the same as that required for a maximum change in the decrease of leucocytes.

Sodium bicarbonate injected into the peritoneal cavity is followed by results identical with those observed following exposure to x-rays. The maximum changes occur in a shorter time following bicarbonate injection, but the relation between chemical and morphological changes are the same.

We consider this analogy to be an important one in that it is suggestive of a relationship between the effect of salt and the effect of x-rays.

THE UNSATURATED FATTY ACIDS OF EGG LECITHIN.

By P. A. LEVENE and IDA P. ROLF.

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The work on liver lecithins by Levene and Ingvaldsen[1] and ·by Levene and Simms,[2] has shown the existence of lecithins which are distinguished from one another by the nature of their unsaturated fatty acids. There were isolated from liver lecithin, two unsaturated fatty acids, oleic and arachidonic, as well as two saturated acids, palmitic and stearic. Hence on the basis of the present findings it is permissible to accept the existence in the liver of four lecithins. Further work may lead to the discovery of an even greater number.

The work on the liver lecithins suggested the need of a reinvestigation of the lecithins of other organs with a view of finding in them also lecithins containing acids of a higher order of unsaturation than that of oleic acid.

The present communication contains the report of the work on egg lecithin. This "lecithin" also was found to contain more than one unsaturated fatty acid. Three acids were isolated; namely, oleic, linoleic, and arachidonic. Oleic acid was identified by its iodine number and by its hydrogenation product, stearic acid. Linoleic acid was isolated in small quantities only and was identified as its tetrabromide. Arachidonic acid was identified by its octabromide and by its hydrogenation product, arachidic acid.

Comparing the mixed lecithins of liver and those of the egg yolk, one is struck by the difference in the proportions of the individual forms in them. The liver lecithin contains a very large proportion of the forms with highly unsaturated fatty acids, whereas in the egg yolk the proportion of the latter is small. Thus, the iodine numbers of the cadmium chloride salts from liver lecithin varied between 59 and 84, whereas those from egg lecithins varied between 30 and 54.

[1] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

[2] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, xlviii, 185.

It is noteworthy that individual samples of egg "lecithin" differed considerably from one another. An effort is being made to develop a method for fractionation of individual lecithins.

<div align="center">EXPERIMENTAL.</div>

A. Isolation of the Unsaturated Acids.

The source of the unsaturated acids was "lecithin cadmium chloride" free from amino-containing impurities. These cadmium chloride salts were prepared from both the acetone and ether extracts of egg powder and were purified by the methods described in previous papers.[3] Decided variation was observed among individual samples obtained either from different lots of egg powder or by different methods of extraction. However, it was not possible to establish a definite relationship between the composition of the material and these variable factors. The fatty acids were liberated by hydrolyzing the cadmium chloride salt for 6 hours with a 10 per cent solution of hydrochloric acid. On cooling, the mixed fatty acids separated as a solid cake. They were then dissolved in ether and thoroughly washed with water. The iodine numbers (method of Wijs) of several such whole fatty acids, are given below.

These acids were derived from salts which were obtained from different lots of egg powder, as well as by varying the methods of extraction.

No. 123 (from an "acetone extract"). 0.3114 gm. substance absorbed 0.2839 gm. iodine corresponding to an iodine number of 91.

No. 117 (from an "ethereal extract"). 0.3465 gm. substance absorbed 0.2839 gm. iodine corresponding to an iodine number of 82.

No. 301 (from an "ethereal extract"). 0.2798 gm. substance absorbed 0.2102 gm. iodine corresponding to an iodine number of 75.

After drying the ethereal solution and concentrating the solvent, a crude separation of the saturated from the unsaturated acids was effected by crystallization from acetone. The mother liquor contains all the unsaturated, with a slight admixture of saturated acids. A more complete separation was accomplished through the insolubility of the barium and lead salts of the saturated acids in ether.

[3] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlvi, 195.

B. Separation of Acids of Varying Degrees of Unsaturation.

Two methods were used in an attempt to separate the constituent acids. The first procedure was based upon the greater solubility of the barium salts of the more highly unsaturated fatty acids in a benzene-alcohol mixture.[4] For this purpose $Ba(OH)_2$ was added to the mixed acids in a methyl alcoholic solution, until the solution was just alkaline to phenolphthalein. The mother liquor was decanted from the gummy precipitate, and concentrated to dryness under diminished pressure. The residue, combined with the original precipitate, was dissolved in a mixture of warm benzene and 5 per cent by volume of 95 per cent ethyl alcohol. To this, after cooling, an additional equal volume of ether was added. On standing over night in the ice box, barium oleate, together with the barium salts of any unsaturated acids present, was precipitated and was easily removed by filtration.

C. Purification of Oleic Acid.

The precipitated barium salts were decomposed with hydrochloric acid, and the liberated acids converted into their lead salts. The solubility of the lead salts of the unsaturated acids in cold ether, permitted the removal by filtration of the last traces of saturated acids. The unsaturated acid, obtained from its lead salt by treatment with hydrochloric acid, was a light yellow liquid which had an iodine value of 89, corresponding to oleic acid.

No. 307.   0.2578 gm. substance absorbed 0.2283 gm. iodine.
$C_{18}H_{34}O_2$.   Calculated.   Iodine value 90.
        Found.   "   "   89.

After hydrogenation by Paal's method the resulting saturated acid (No. 307) was analyzed and determinations of the molecular weight and melting point were made.

All samples of saturated fatty acids reported in this paper were dried by fusion on an electric hot-plate, and the material used for combustion was remelted under diminished pressure at the temperature of xylene vapor, until constant weight was obtained. The

[4] Farnsteiner, K. Z. Untersuch. Nahrungs-u. Genussmittel., 1899, ii, 1.

bromo acids were dried under diminished pressure at the temperature of boiling chloroform.

The melting points as recorded are corrected, and were taken at such a rate that the time per degree rise in temperature was 6 seconds. The molecular weights were calculated by the titration of approximately 1 gm. of acid, dissolved in 10 cc. of toluene and 25 cc. of methyl alcohol (neutral to phenolphthalein) with 0.5 N NaOH, using phenolphthalein as an indicator.

No. 307.   0.1000 gm. substance: 0.1176 gm. H_2O and 0.2786 gm. CO_2.
             0.8465 "   "   required for neutralization 6.00 cc.   0.5 N NaOH.
$C_{18}H_{36}O_2$. Calculated.   C 75.98, H 12.72. (Molecular weight = 284.
                                                    Melting point = 70–71°C.)
             Found.   C 75.97, H 13.14. (Molecular weight = 282.
                                                    Melting point = 71°C.)

D. Acids of a Higher Degree of Unsaturation.

The barium salts soluble in the mixture of benzene-alcohol-ether, as described under Section B, were decomposed with hydrochloric acid. The liberated acid, a dark brown liquid, had an iodine value of 165.

0.2055 gm. substance absorbed 0.3392 gm. iodine.

This acid after reduction by Paal's method gave the following analytical data.

No. 309.   0.1008 gm. substance: 0.1192 gm. H_2O and 0.2832 gm. CO_2.
             1.7580 "   "   required for neutralization 11.70 cc.   0.5 N NaOH.
$C_{18}H_{36}O_2$. Calculated.   C 75.98, H 12.72. (Molecular weight = 284.
                                                    Melting point = 70–71°C.)
$C_{20}H_{40}O_2$. Calculated.   C 76.95, H 12.81. (Molecular weight = 312.
                                                    Melting point = 75–77°C.)
             Found.   C 76.61, H 13.21. (Molecular weight = 299.
                                                    Melting point = 66–67°C.)

E. Fractionation of the Methyl Esters.

For the fractional distillation of the methyl esters the crude unsaturated acids were converted into their lead salts. The ether-soluble fraction was decomposed with hydrochloric acid and the free acids were esterified. These methyl esters were then saturated

with hydrogen by Paal's method and again esterified. The recrystallized esters were distilled at a pressure of 3 mm. into four fractions, each of which was then saponified and the free acid purified by conversion through the lead salt. The molecular weights, melting points, and analyses of the respective acids are recorded below.

No. 312.　　0.1002 gm. substance: 0.1162 gm. H_2O and 0.2750 gm. CO_2.
　　　　　　1.0132　　"　　　"　　required for neutralization 7.18 cc. 0.5 N HaOH.
No. 314.　　0.1012 gm. substance: 0.1182 gm. H_2O and 0.2820 gm. CO_2.
　　　　　　1.0216　　"　　　"　　required for neutralization 7.22 cc. 0.5 N NaOH.
No. 315.　　0.1000 gm. substance: 0.1144 gm. H_2O and 0.2762 gm. CO_2.
No. 316.　　0.0970 gm. substance: 0.1172 gm. H_2O and 0.2698 gm. CO_2.
　　　　　　0.7858　　"　　　"　　required for neutralization 5.15 cc. 0.5 N HaOH.
$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.76. (Molecular weight = 284.
　　　　　　　　　　　　　　　　Melting point = 70–71°C.)
$C_{20}H_{40}O_2$. Calculated. C 76.95, H 12.98. (Molecular weight = 312.
　　　　　　　　　　　　　　　　Melting point = 75–77°C.)

	No.	Boiling point of ester. Pressure 3 mm.	Analysis of acid.		Weight of ester.	Molecular weight of acid.	Melting point of acid.
			C	H			
		°C.			gm.		°C.
Found.	312	178–182	74.88	12.98	3.5	282	70–71
"	314	175–182	75.98	13.07	5.3	283	70–71
"	315	177–185	75.31	12.80	4.6		62–63
"	316	184–195	77.03	13.30	2.5	308	75

F. Bromine Addition Products of the More Highly Unsaturated Acids.

The acids, whose barium salts were soluble in benzene-alcohol-ether, were dissolved in 10 parts of glacial acetic acid. To the cooled solution a 10 per cent solution of bromine in glacial acetic acid was added very gradually. The bromination was accompanied by a characteristic color change and the appearance of a very finely divided amorphous precipitate. After standing over night the latter was separated by centrifugalization and extracted with warm ether until further extracts were colorless. On drying, the material darkened slightly. When heated in an open tube it browned gradually above 215°, and contracted at 250°. It neither melted nor did it decompose with gas evolution. In a closed tube, this material after recrystallization from glacial acetic acid, contracted at 250°. It darkened decidedly above this point, and sintered very definitely at 255°.

By the hydrolysis of 380 gm. of a lecithin cadmium chloride (No. 123) whose iodine value was 54, mixed acids were obtained which had an iodine value of 91. From these, 60 gm. of acids, whose barium salts were soluble in a benzene-alcohol mixture, were isolated. These acids on bromination yielded 4.5 gm. of an octabromide insoluble in ether, but very slightly soluble in hot glacial acetic acid.

Analyses of two products obtained in this manner are given below:

No. 336. 0.0932 gm. substance: 0.3000 gm. H_2O and 0.0864 gm. CO_2.
          0.2068 "   "   0.3304 " AgBr.
No. 304. 0.1024 gm. substance: 0.0326 gm. H_2O and 0.0942 gm. CO_2.
No. 362. 0.1846 "   "   0.2920 " AgBr.
$C_{20}H_{32}O_2Br_8$. Calculated. C 25.43, H 3.42, Br 67.72.
No. 336. Found.   " 25.28, " 3.60, " 67.99.
No. 304.   "   " 25.08, " 3.56, " 67.32.

The bromination liquor, after the removal of the octabromide was concentrated to dryness under diminished pressure. The residual syrup was dissolved in ether and washed with sodium thiosulfate. From the concentrated ethereal solution, aggregates of spear-like needles were deposited. From the bromination of the acids of No. 123, details of which were given above, about 8 gm. of this crude material were obtained. This material was readily soluble in ether and absolute alcohol, but was insoluble in gasoline. After repeated recrystallization from various solvents, two samples, obtained respectively from an "ether" and an "acetone" extract, gave the following analyses, molecular weights, and melting points.

No. 355. 0.1034 gm. substance: 0.0512 gm. H_2O and 0.1368 gm. CO_2.
          0.2012 "   "   used for Carius determination: 0.2596 gm. AgBr.
          0.8401 "   "   requires for neutralization 13.70 cc. 0.1 N NaOH,
              corresponding to a molecular weight of 617.
In an open tube it softened at 115° and melted at 117–119°C.
No. 307. 0.1012 gm. substance: 0.0476 gm. H_2O and 0.1340 gm. CO_2.
          0.2010 "   "   0.2526 " AgBr.
          0.5412 "   "   required for neutralization 9.00 cc. 0.1 N NaOH,
              corresponding to a molecular weight of 601.
          Melted in an open tube at 116–117°C.
$C_{18}H_{32}O_2Br_4$. Calculated. C 36.01, H 5.38, Br 53.28. (Molecular weight = 600.)
No. 355. Found.   C 36.08, H 5.54, Br 54.22. (Molecular weight = 617.
                                              Melting point = 115–116°C.)
No. 307. Found.   C. 36.10, H 5.22, Br 53.48. (Molecular weight = 601.
                  .   Melting point = 115–116°C.)

Tetrabromostearic acid was prepared by brominating the acids of cottonseed oil. This material when heated, contracted at 114° and melted at 115–116°C. When mixed with these tetrabromo acids from lecithin, no depression of its melting point was apparent.

SYNTHESES IN THE CINCHONA SERIES.

VII. 5, 8-DIAMINO-DIHYDROQUININE AND 5,8-DIAMINO-6-METHOXY-QUINOLINE AND THEIR CONVERSION INTO THE CORRESPONDING AMINOHYDROXY AND DIHYDROXY BASES.

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In a former communication[1] amino-azo dyes prepared from 5-amino-dihydroquinine and the analogous 5-amino-6-methoxyquinoline were described. These substances were shown to be easily converted by acids into the corresponding hydroxyazo dyes. By reduction of 8-(*p*-sulfophenylazo)-dihydroquinine we have now prepared 5,8-diamino-dihydroquinine. The base itself is a yellow amorphous powder which crystallizes with great difficulty and rapidly undergoes alteration on exposure to light and air. Like 5-amino-quinoline, it dissolves in dilute acids to form red solutions and yields crystalline salts of which the vermilion tetrahydrobromide and the brown basic sulfate have been studied.

As in the parent amino-azo dyes, it was not surprising to find the amino group here also easily replaceable by hydroxyl. In fact, this occurs so readily that it was at first found difficult to select conditions for the isolation of the polyacid salts. Lability, however, was not confined to the amino group in Position 5, but was shown also by that in Position 8, for on long standing in the cold or on boiling with 1:1 hydrochloric acid 5, 8-dihydroxy-dihydroquinine was formed. This substance was isolated as the beautifully crystalline red dihydrochloride which forms orange-red solutions. The instability of the free base prevented its isolation in crystalline form.

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[1] THIS JOURNAL, **42**, 2278 (1920).

From numerous tests that were made, the impression was gained that the amino group in Postion 5 was the more readily replaced and that 8-amino-5-hydroxy-dihydroquinine was formed as an intermediate product.   Unfortunately, the isolation of this substance from the reaction mixture was rendered very difficult by its properties.   We have, however, obtained indirect evidence of its formation by a parallel study of the effect of acids on diamino-methoxyquinoline as described below.   5-Hydroxy-8-amino-dihydroquinine was, however, obtained directly as a tin double salt by reduction of 5-hydroxy-8-phenylazo-dihydroquinine,[2] but the instability of the free aminophenol and the solubility of its simple salts prevented their isolation and study.   Like the preceding compounds, solutions of the latter are orange-red.

By boiling diamino-dihydroquinine with conc. hydrobromic acid, which should demethylate as well as desaminate it, yellow needles of an easily soluble hydrobromide of what is probably the 5,6,8-trihydroxy dihydrobromide were obtained mixed with ammonium bromide, but other work intervened to prevent its further study.   We hope to complete this study at a later date, as well as the alkylation of the di- and trihydroxy-dihydroquinines.

Parallel with the above studies, 5,8-diamino-6-methoxyquinoline was prepared from the corresponding sulfo-phenylazo dye.   The latter was also converted into the 5-hydroxy-8-phenylazo dye. from which 8-amino-5-hydroxy-6-methoxyquinoline was obtained on reduction. On warming diamino-methoxyquinoline with 10% hydrochloric acid, it was possible to isolate as the main product of the reaction an amino hydroxy compound identical with that of known composition obtained from the above hydroxyazo dye.   From this it is evident that the amino group in Position 5 is more labile than that in Position 8.

Finally, by boiling the diamino compound with stronger hydrochloric acid both amino groups were replaced, with the production of 5,8-dihydroxy-6-methoxyquinoline.

The quinoline compounds proved to be more stable than the dihydroquinine derivatives and were readily obtained in crystalline form.

It was also of interest to determine whether the methoxy group in

[2] Ref. 1, p. 2280.

Position 6 contributed to the lability of these amino groups, since it had been already ascertained in our previous work with the amino-azo dyes that those obtained from 5-aminoquinoline itself were much more resistant to the action of acids than the 6-methoxy compounds. 5,8-Diaminoquinoline proved to be even more resistant than its parent azo dye, since boiling it for many hours with hydrochloric acid resulted in the cleavage of but a trace of ammonia. The substitution of Position 6 in these compounds is therefore a determining factor as regards the replaceability of the amino group by hydroxyl by the action of boiling acids.

A. DERIVATIVES OF DIHYDROQUININE.

5, 8-Diamino-dihydroquinine.—Sixteen g. of 5-amino-8-(*p*-sulfo-phenylazo)-dihydroquinine[3] were dissolved in 80 cc. of 50% acetic acid and treated with 15 g. of stannous chloride in 50 cc of 1:1 hydrochloric acid. A gelatinous mass of the tin compound of the unreduced dye was first formed, and this was rapidly dissolved as it was stirred and the reduction proceeded The mixture warmed considerably and changed to a deep brownish-red solution from which the yellow tin double salt of the diamino compound commenced to separate The mixture was diluted with water and poured into ice and an excess of alkali, the diamino-dihydroquinine precipitating as yellow flocks. The collected precipitate was washed thoroughly with water, during which the exposed portion darkened perceptibly. Dried in a desiccator it formed a tan colored friable mass The crude product was dissolved in a small volume of benzene, and the deep brown-olive solution cleared with bone black and filtered as quickly as possible The deeply colored filtrate was treated with ligroin in amount sufficient to precipitate the product as a brown paste which rapidly hardened to a brittle mass. This was filtered quickly, washed with ligroin and dried in a desiccator containing paraffin, during which it lightened in color to a brown-yellow shade. The yield was 8 g. On exposure to light and air the base gradually darkened. All attempts to crystallize the amorphous substance have proved futile, except in one case in which we observed that during the collection of the base from the benzene and ligroin mixture, the mother liquor slowly deposited more of the substance which appeared under the microscope as minute yellow needles. These possessed all the properties of the former product with the exception of the melting point but, unfortunately, the amount was too small for analysis and its use as seeding material in attempts to crystallize the amorphous base was without result.

The amorphous base darkens above 90°, gradually shrinks together and melts from 125° to 140° with decomposition. The crystalline material, however, melted at 85 to 90° to a dark tar. The amorphous base is easily soluble in the usual organic

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[3] Ref. 1, p. 2281.

solvents except ligroin, forming dark brownish-red solutions. In dilute acids it forms deep brown-red solutions which, on standing or more rapidly on heating, no longer give a precipitate of the base when treated with an excess of alkali, but yield solutions which smell strongly of ammonia.

Analyses. Calc. for $C_{20}H_{28}O_2N_4$: C, 67.40; H, 7.86; N, 15.71. Found: C, 68.00; H, 7.82; N, 15.59.

THE TETRAHYDROBROMIDE.—The amino-azo dye was also readily reduced to the diamino base as follows. Five and a half g. of the dye were dissolved in a mixture of 55 cc. of alcohol and 55 cc. of conc. ammonia, and the deep red solution was saturated with hydrogen sulfide. The color changed to a deep yellow-brown and, as the base did not separate, the mixture was poured into a separatory funnel, shaken with ether, and then without removing the ether an excess of ammonia was added and the mixture again shaken. This procedure removed free hydrogen sulfide and avoided the formation of pasty lumps of the base which are difficult to disintegrate and dissolve. After drying the ether extract and concentrating, the base was left as a dark syrup. On dissolving this in a small volume of cold water and sufficient cold hydrobromic acid to make the solution acid to congo red, the addition of sodium bromide accompanied by rubbing caused 5 g. of lustrous golden scales to separate. These were filtered off and washed with acetone, the color changing to an orange-red. A solution of the salt in 12 cc. of warm water was cooled and treated with sufficient strong hydrobromic acid to cause crystallization. The salt separated slowly as vermilion needles and prisms which were air-dried. It is easily soluble in water and in alcohol, forming deep red solutions. The analytical results were not all that could be desired, but with a substance so sensitive to the action of acids and so easily hydrolyzed we were unable to improve upon them.

Analyses. Calc. for $C_{20}H_{28}O_2N_4.4HBr$: N, 8.24; Br, 47.04. Found, air-dry: N, 8.00; Br, 49.29. Salt dried in desiccator over H_2SO_4: N, 8.47; Br, 41.80.

THE SULFATE.—The brown-red ether extract of the base obtained by the reduction of 16 g. of dye by either method was washed with water and then shaken out with 50 cc. of N acetic acid. The deep brown-red aqueous layer was filtered rapidly and treated with saturated ammonium sulfate solution. On rubbing, the sulfate crystallized as a yellow-brown powder consisting of microscopic 6-sided polyhedra, while the mother liquor retained dark impurities. The yield was excellent. The salt was washed with ice water and recrystallized in small portions by dissolving it in hot water and adding a little ammonium sulfate solution to the dark yellow-brown solution. When cooled and rubbed the solution deposited the salt as a brown powder consisting of globules or hexagonal crystals which darken rapidly and undergo decomposition. The anhydrous substance darkens above 160° and melts and decomposes at 220–225°. ·

Although the salt is obtained from an acid solution, it is rather surprising that, judged by analytical data, the substance is a basic sulfate.

In the analysis of the substance, water was determined by drying it over sulfuric acid at room temperature, and sulfur by the Carius method.

Analyses. Calc. for $(C_{20}H_{28}O_2N_4)_2.H_2SO_4.5H_2O$: H_2O, 10.00. Found: H_2O, 10.68. Calc. for $(C_{20}H_{28}O_2N_4)_2.H_2SO_4$: C, 59.22; H, 7.16; N, 13.82; S, 3.96. Found: C, 58.50; H, 7.21; N, 13.87; S, 4.35.

5-Hydroxy-8-amino-dihydroquinine (tin double salt).—Fourteen and a half g. of 5-hydroxy-8-phenylazo-dihydroquinine[2] were dissolved in 100 cc. of alcohol by the addition of a few cubic centimeters of acetic acid, warmed, shaken vigorously and treated as quickly as possible with a warm solution of 18 g. of stannous chloride in 100 cc. of 10% hydrochloric acid. The thick paste which first formed dissolved rapidly as the reduction proceeded, giving a deep red solution from which a good yield of the tin double salt of the aminophenol quickly separated as lustrous golden-yellow needles and leaflets. These were filtered, washed with 10% hydrochloric acid, and recrystallized from 50% acetic acid, from which the salt separated in the same form. The substance was dried in a desiccator over sulfuric acid and sodium hydroxide. The salt darkens above 200°, but does not melt when heated to 280°. It is fairly soluble in water or dilute alcohol, especially on warming, yielding orange-red solutions. It is practically insoluble in alcohol or acetone. It dissolves in alkali, forming a clear, light brown solution which deposits light colored flocks on standing. Ferric chloride, added to the aqueous solution, gives a light green color changing to a deep emerald green when sodium acetate is added.

Analyses. Calc. for $C_{20}H_{27}O_3N_3.2HCl.SnCl_4$: C, 34.74; H, 4.24; N, 6.08. Found: C, 35.55; H, 4.47; N, 6.13.

When an aqueous suspension of the tin double salt was decomposed with hydrogen sulfide the filtrate consisted of a red solution of the aminophenol hydrochloride. Attempts made to obtain the crystalline salt failed since this proved too soluble for isolation, and manipulation was rendered difficult by the ease with which the amino group was replaced by hydroxyl under the influence of acid. In a number of instances, in which the solution stood for a long time in the refrigerator, a well-defined hydrochloride crystallized, but analysis showed this salt to be the dihydrochloride of dihydroxy-dihydroquinine. Likewise all attempts to obtain the free base were unsuccessful because of its instability.

5,8-Dihydroxy-dihydroquinine Dihydrochloride.—Diamino-dihydroquinine obtained from 24 g. of the *p*-sulfo-phenylazo dye was boiled with 5 parts of 1:1 hydrochloric acid for 3 hours, and the resulting deep red solution concentrated to smaller volume and chilled, rosets of red silky needles soon filling the liquid. The salt, washed with 10% hydrochloric acid, was recrystallized from this solvent and formed rosets of vermilion needles which were collected, washed with 10% hydrochloric acid, and air-dried. The yield was 5.5 g. The salt is easily soluble in water or alcohol, forming an orange-red solution. It is less soluble in dil. hydrochloric acid or in salt solution, and insoluble in acetone. When anhydrous, it decomposes at 208–211°. Sodium carbonate and dil. aqueous ammonia added to its solution precipitate the free base as yellow amorphous flocks which rapidly turn green on exposure to air and finally become brown. It is soluble in alkali and excess of ammonia to form brown solutions which gradually deepen in color. Ferric chloride gives a light

brown color which changes to a brown-olive when sodium acetate is added.   The addition of hydrobromic acid or sodium bromide to the solution of the salt causes the separation of the hydrobromide as rosets of flat, red needles.

The salt was also obtained when acid solutions of amino-hydroxy-dihydroquinine were allowed to stand for a long time.

Analyses.   Calc. for $C_{20}H_{26}O_4N_2.2HCl.4H_2O$: H_2O, 14.31.   Found: H_2O, 15.10. Calc. for $C_{20}H_{26}O_4N_2.2HCl$: N, 6.49; Cl, 16.43.   Found: N, 6.57; Cl, 16.25.

Because of the instability of the free base we have been unable to obtain it in crystalline form.

B. QUINOLINE DERIVATIVES.

5-Amino-6-methoxy-8-(*p*-sulfo-phenylazo) quinoline.—Diazotized sulfanilic acid was coupled in the usual way with 5-amino-6-methoxyquinoline[4] in dil. acetic acid containing sufficient sodium acetate to act as buffer.   A deep purple solution formed, accompanied by a tar which crystallized when alcohol was added.   When dissolved in 50% alcohol with the aid of ammonia, and heated, re-acidifying the solution with acetic acid, the acid separated as flat, brown, microscopic needles which did not melt when heated to 295.°   It is almost insoluble in the usual neutral solvents. The solution in dil. alkali is orange-red, and purple in dil. acids, while in conc. sulfuric acid it gives a red solution which appears purple in thin layers.

Analysis.   Calc. for $C_{16}H_{14}O_4N_4S$: N, 15.63.   Found: N, 16.04.

5, 8-Diamino-6-methoxyquinoline.—Thirty-two g. of the sulfo-phenylazo dye dissolved in 300 cc. of 10% ammonia were saturated with hydrogen sulfide.   Decolorization occurred rapidly, with deposition of crystals of the deep olive-brown diamino base.   The mixture was made distinctly ammoniacal, filtered, and washed with water. The yield was 15 g.   Extraction of the mother liquor with ether gave an additional gram.   Recrystallization from toluene with the addition of bone black gave lustrous golden leaflets which are fairly stable when pure, but gradually turn olive colored when moist, due to oxidation.   When rapidly heated, the base darkens above 155°, sinters, and then melts at 163–164° to form a dark tar.   It is appreciably soluble in acetone and chloroform, especially on warming, and in hot alcohol, benzene or toluene, and only sparingly soluble in ether, to form yellow solutions which darken to a brown-olive color on standing.   The solution in dil. acid is a reddish-orange, resembling the corresponding dihydroquinine derivative, and in conc. sulfuric acid the color is a faint yellow.   A concentrated solution in 10% hydrochloric acid deposits delicate yellow needles of the polyhydrochloride on chilling.   The solution in dil. acetic acid turns a light green when treated with ferric chloride, changing to a deep emerald green when sodium acetate is added.

Analysis.   Calc. for $C_{10}H_{11}ON_3$: N, 22.22.   Found: N, 22.34.

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[4] Ref. 1, p. 2285.

8-Amino-5-hydroxy-6-methoxyquinoline.—Three g. of diamino-6-methoxy-quinoline were dissolved in 30 cc. of 10% hydrochloric acid, warmed for 30 minutes on the water-bath, then diluted with water, and boiled free from air. The solution was chilled and neutralized with a considerable excess of sodium acetate solution. When rubbed the solution yielded 2 g. of the aminophenol as a yellow powder which quickly turned to an olive color on the surface because of oxidation. It was recrystallized by dissolving it in a considerable amount of hot toluene, filtering rapidly, cooling, and again filtering rapidly from green amorphous flocks which had separated. On scratching, the yellow filtrate yielded aggregates of yellow microscopic leaflets or stout crystals which darkened to an olive color when exposed to the air. It darkened above 130° and slowly melted to a dark mass at 180–182° with preliminary sinter-ing. It is appreciably soluble in methyl alcohol, acetone, and ether, and in hot benzene or toluene. It crystallizes from hot alcohol as stout microscopic crystals and dissolves in dil. acids with the formation of an orange-red solution. Addition of ferric chloride to a solution, followed by sodium acetate, gave a deep emerald-green color. The solution in alkali, at first yellow in color, changes almost instantly to green.

Analysis. Calc. for $C_{10}H_{10}O_2N_2$: N, 14.73. Found: N, 14.60.

This compound proved to be identical with the substance obtained as follows. Fourteen g. of 5-hydroxy-6-methoxy-8-phenylazoquinoline, described below, were dissolved in 140 cc. of 10% ammonia, and hydrogen sulfide was passed in until the deep purple liquid changed to a brown, with deposition of glistening crystals of the 8-amino-5-hydroxy-6-methoxyquinoline. The yield was 6 g. Recrystallization from toluene, as previously described, gave a yellow substance which agreed in all properties with the aminophenol described above. This was confirmed by a mixed melting point determination.

Analysis. Calc. for $C_{10}H_{10}O_2N_2$: N, 14.73. Found: N, 14.63.

5-Hydroxy-6-methoxy-8-(p-sulfo-phenylazo)quinoline.—Because of the insol-ubility of the amino-azo dye described above, its conversion into the hydroxyazo dye could not be accomplished conveniently in alcoholic solution as in previous instances but was carried out as follows. Sixteen g. of the amino-azo dye were suspended in 160 cc. of hot glacial acetic acid and then 160 cc. of hot 1:1 hydrochloric acid were added. As the deep purple paste of the hydrochloride which formed was heated on the water-bath it gradually dissolved, and after 10 minutes the solution turned a deep orange-red and deposited the hydroxyazo dye almost quantitatively. Redissolved in hot 50% alcohol with the aid of ammonia and re-acidified with hydrochloric acid it formed a purple powder which appeared under the microscope as rosets of brown platelets. It is practically insoluble in neutral solvents and does not melt when heated to 290°.

Analyses. Calc. for $C_{16}H_{13}O_5N_3S.H_2O$: H_2O, 4.77. Found: H_2O, 5.51. Calc. for $C_{16}H_{13}O_5N_3S$: N, 11.68. Found: N, 11.78.

5,8-Dihydroxy-6-methoxyquinoline.—When 5 g. of diamino-methoxyquinoline were boiled for 3 hours in 1:1 hydrochloric acid and then chilled, red-brown crystals

of the dihydroxy hydrochloride slowly separated. This salt was washed with the acid, dissolved in water, and treated with an excess of sodium acetate. On rubbing, 3 g. of the base quickly separated as yellow microscopic prisms which became purple on exposure. It was recrystallized from about 40 parts of alcohol and then from toluene, forming lustrous yellow leaflets and needles melting at 195–197° with slight preliminary softening and darkening. It is sparingly soluble in the cold in methyl and ethyl alcohols and in acetone, but more readily soluble if the solvent is warmed. It dissolves in dil. acids with the formation of a brown-orange colored solution and in conc. sulfuric acid with an orange-yellow color. The solution in alkali is brown and the shade deepens on standing, while an alcoholic solution gives an olive color with ferric chloride.

Analyses. Calc. for $C_{10}H_9O_2N$: C, 62.80; H, 4.71; N, 7.33. Found: C, 62.90; H, 4.72; N, 7.81.

Action of Acids on 5,8-Diaminoquinoline.—Two and a half g. of diaminoquinoline were boiled 3 hours with 1:1 hydrochloric acid, a portion of the hydrochloride remaining undissolved throughout. From the collected salt almost all of the base was recovered unchanged. When the mother liquor was rendered alkaline, it showed the presence of a trace of hydroxy compound by the deposition of green flocks, but the odor of ammonia was scarcely detectable on boiling.

SUMMARY.

Like the amino groups in the amino-azo dyes derived from 5-amino-dihydroquinine and 5-amino-6-methoxyquinoline, those in the 5, 8-diamino-compounds obtained from the dyes by reduction are easily replaceable by hydroxyl. A number of the intermediate and end products of this transformation are described.

SYNTHESES IN THE CINCHONA SERIES.

VIII. HYDROGENATION OF DIHYDROCINCHONINE, CINCHONINE AND DIHYDROQUININE.

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The hydrogenation of the cinchona alkaloids has been studied by many workers in the past. Since the attempts of others by different methods had yielded substances of indefinite character, Konek von Norwall,[1] and Lippmann and Fleissner[2] applied the Ladenburg method of reduction with sodium and alcohol with the idea of reducing the quinoline portion of the substance. Lippmann and Fleissner obtained from quinine a strongly basic, thick oil or resin which gave the color reactions of a tetrahydroquinoline derivative but from which no crystalline compounds were obtained, with the exception of a chloroplatinate. Konek von Norwall reduced cinchonine with sodium and ethyl alcohol to an amorphous non-crystallizable substance which he believed to be a dihydrocinchonine. He subsequently substituted amyl alcohol as the solvent and then obtained a product which, although amorphous as the free base, was readily converted by nitrous acid into a crystalline salt which he described as the nitrite of a nitrosotetrahydrocinchonine. Quinine, quinidine and cinchonidine were then found to yield similar products.

In 1901 Tafel[3] noted that by the use of his electrolytic reduction method on the cinchona alkaloids 4 hydrogen atoms were absorbed, the product, however, being non-crystalline. More recently Freund

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*Presented at the Annual Meeting of the American Chemical Society, New York, September, 1921.

[1] von Norwall, *Monatsh.*, 16, 630 (1895).

[2] Lippmann and Fleissner, *ibid.*, 16, 321 (1895); *Ber.*, 28, 1637 (1895); 29, 801 (1896.)

[3] Tafel, *Ber.*, 34, 3299 (1901).

and Bredenberg[4] repeated Tafel's work and obtained from cinchonine a compound in 40% yield in which one hydrogen molecule entered the quinoline nucleus, with simultaneous reduction of the secondary alcoholic group by the other. This substance was identified as a Q-dihydro-desoxycinchonine or Q-dihydrocinchonane.[5] A by-product of the reaction was an oily non-crystalline substance possessing the same composition and which these workers considered to be an isomer and designated as isodihydro-desoxy-cinchonine. With the reduction of the secondary hydroxyl group to hydrogen, the asymmetry of the carbon atom
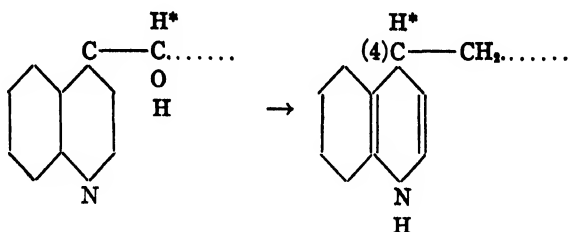
connecting the quinoline and the quinuclidine nuclei is destroyed, while the addition of the two hydrogen atoms to the quinoline nucleus creates a new asymmetric carbon at Position 4 which would explain the production of two isomeric bases. On reduction of the crystalline base with sodium and amyl alcohol an oily tetrahydro-desoxycinchonine (tetrahydrocinchonane) was obtained which yielded on hydrogenation with palladium and hydrogen an oily hexahydro-cinchonane, both bases forming crystalline salts.

A year later Skita and Brunner[6] succeeded in reducing the cinchona alkaloids with a large excess of platinum black and hydrogen under pressure to high-melting crystalline hexahydro alkaloids and finally to the dodecahydro compounds. In the case of the hexahydro alkaloids, however, they make no mention of the occurrence of isomers resulting from the new asymmetry of carbon (4).

In connection with our synthetic studies in the cinchona group we were led to seek for a less costly and cumbersome method for their

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[4] Freund and Bredenberg, *Ann.*, 407, 43 (1915).

[5] For the meaning of the suffix -*ane*, cf. THIS JOURNAL, 42, 1489 (1920).

[6] Skita and Brunner, *Ber.*, 49, 1597 (1916).

reduction than that devised by Skita and Brunner. Because of the rather indefinite results of Konek von Norwall and Lippmann and Fleissner, it occurred to us that, by using the dihydro alkaloids as starting material, sodium and amyl alcohol should yield products the same as those described by Skita and Brunner. To our surprise, however, dihydrocinchonine yielded an oil which was found to be a mixture of different bases. On converting this mixture into the dihydrochlorides in absolute alcoholic solution, it was found possible by fractional crystallization to isolate three different salts. The least soluble of these was obtained in about 25% yield and proved to be the dihydrochloride of a hexahydrocinchonane[7] which yielded a crystalline

[7] After this communication had been sent to the editor, papers by G. Giemsa and J. Halberkann [*Ber.*, 54, 1167, 1189 (1921)] appeared in which these authors criticize our use of the ending "ane" as a simplification of the term "desoxy" alkaloids on the ground that such a name should just as properly belong to the "still hypothetical reduction products of hydroquinene" and consequently necessitate the confusing use of prefixes α-, β-, etc. These authors apparently have failed to realize that if Rabe's views on the stereochemical relationships of the cinchona alkaloids are correct (and they are supported by excellent evidence) the reduction of hydroquinene should yield a mixture of what we have called dihydroquinane (dihydrodesoxyquinine and dihydroquinidane (dihydro-desoxyquinidane), a third substance being impossible. In spite of their objections to this terminology, they have applied it to their own apparently isomeric substances, designating as hydrocupreane and hydroquinane the substances prepared by them by diazotization of the 5-amino alkaloids. They justify this on the ground that their substances are produced by a method so gentle as to make unlikely the occurrence of optical isomerization in the reduction of the secondary alcoholic group to CH_2, whereas the reaction involving the use of phosphorus pentachloride for the preparation of the desoxy alkaloids over the chloro compounds is more apt to affect the steric relationship of these to the parent alkaloids.

These statements are incompatible with Rabe's views, and unless Giemsa and Halberkann's "hydrocupreane" had undergone optical or other intramolecular rearrangement it should have been identical with our dihydrocupreane. Since it has been shown to be different it must be concluded that their substances, the products of a little understood reaction, are the result of some such rearrangement.

Finally, the possibility of any isomerization with phosphorus pentachloride seems most remote since the direct reduction of dihydrocinchonine with sodium or zinc as described in this paper yields the same α- and β-hexahydrocinchonanes as those obtained by reduction of dihydrocinchonane which was prepared over the chloro compound. We see no reason, therefore, for discontinuing the use of the terminology which we have begun.

base melting at 106–7°. The most soluble salt proved also to be the dihydrochloride of a hexahydrocinchonane and was obtained in about equal amount. This salt likewise yielded a crystalline base melting at 106–106.5°. We have designated the former base α- and the latter as β-hexahydrocinchonane. These substances are unquestionably epimers, both being formed when carbon atom (4) of the quinoline ring becomes asymmetric, just as was assumed by Freund and Bredenberg in the electrolytic reduction of cinchonine. That these substances are tetrahydroquinoline derivatives was shown by the fact that they form stable dihydrochlorides, nitrosamines, N-acyl derivatives and phenylazo dyes, the last of which was obtained in crystalline form only in the case of the α-compound. They also give the characteristic color reactions of tetrahydroquinoline with ferric chloride.

It appeared rather surprising that the reduction of the secondary alcoholic group of cinchonine to the CH_2 group should have occurred here as well as in the electrolytic reduction. To prove the correctness of the findings, however, dihydrocinchonine was converted through the chloro compound into dihydrocinchonane, which was in turn reduced with sodium and amyl alcohol; α- and β-hexahydrocinchonane were obtained exclusively. One of these isomers must therefore be the hexahydro-desoxycinchonine described by Freund and Bredenberg but not obtained by them in crystalline form.[8]

The third substance obtained by fractionation of the hydrochlorides was isolated finally as a dihydrobromide. Its elementary composition corresponds to that of a hexahydrocinchonine. Unfortunately, the base could not be obtained crystalline and could not, therefore, be compared with the product of Skita and Brunner. We are, however, of the opinion that our substance is different from theirs. From our

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[8] Since a tetrahydrocinchonane (tetrahydro-desoxycinchonine) which they also describe was found by them to possess $[\alpha]_D$ 64.6°, this substance must therefore be isomeric with the tetrahydrocinchonane prepared by us by the reduction of cinchonine and which is described further on. This base gave $[\alpha]_D$ 209.0°. Accordingly, our β-base should belong to the series of compounds which these workers obtained from their crystalline dihydrocinchonane and should therefore be the same as their amorphous hexahydro-desoxycinchonine. Unfortunately, they did not describe its rotation.

experience and that of Freund and Bredenberg, it is rather strange that Skita and Brunner did not observe the formation of epimeric isomers in the reduction of the alkaloids by their method. Our third substance also gives all of the reactions of a tetrahydroquinoline. Under the conditions employed it would seem, therefore, that the alcoholic group does not suffer reduction in all of the alkaloid used.

To ascertain whether other reducing agents would yield the same compounds, dihydrocinchonine was reduced with zinc and hydrochloric acid; this process yielded an oily base from which an appreciable amount of α-hexahydrocinchonane was isolated by means of the hydrochloride. Since the yield of this product was much poorer than that obtained by the sodium method, the reaction unquestionably proceeded partly in a different sense.

It was then of interest to repeat the experiments of Konek von Norwall with cinchonine itself. Here, also, a viscous sirup was obtained which was likewise found to be a mixture. By conversion into the hydrochloride a crystalline fraction was obtained with little difficulty which yielded a crystalline base melting at 116.5–117.5° and with practically the same rotation given by α-hexahydrocinchonane. This base proved to be α-tetrahydrocinchonane, since on reduction with palladium and hydrogen it yielded the α-hexahydro base. From the mother liquor of the above salt it was found difficult to isolate any other products of the reduction. A clue to their nature was found, however, by reducing the residue with palladium and hydrogen, a process which could reduce only the vinyl side-chain. From the resulting mixture the hydrochlorides of α- and β-hexahydrocinchonane and hexahydrocinchonine dihydrobromide were readily isolated, showing that the original reduction mixture contained besides α-tetrahydrocinchonane, β-tetrahydrocinchonane and tetrahydrocinchonine. The so-called nitroso-tetrahydrocinchonine nitrite described by Konek von Norwall was therefore probably a mixture and not solely, if at all, a derivative of tetrahydrocinchonine.

Finally dihydroquinine was also reduced by this method, but curiously enough the only substance which could be isolated in pure crystalline form and in fair yield was the dihydrochloride of hexahydroquinine. The nature of this salt was established by the preparation of a nitroso and monobenzoyl derivative and the usual color reactions. The base unfortunately could not be obtained in crystalline form.

EXPERIMENTAL.

A. REDUCTION OF DIHYDROCINCHONINE (CINCHOTINE).

100 g. of dihydrocinchonine were dissolved in 2 kg. of boiling dry amyl alcohol and reduced at the boiling temperature with 150 g. of sodium. The reaction required in all from 4 to 6 hours. When the practically colorless solution was treated with water and the amyl alcohol removed with steam, a resinous residue remained which was extracted with ether. Concentration of the dried ethereal solution left an amber colored oil which was dissolved in 1 liter of àbsolute alcohol. A solution of hydrochloric acid in absolute alcohol was added until the reaction became acid to congo red. A copious crop of microscopic needles gradually separated and was collected and washed with cold, acidulated absolute alcohol, forming a faintly bluish-purple mass which weighed 50 g. when dry. Recrystallization from 3 parts of boiling water gave a crop of almost flat, minute, colorless needles which, after they had been washed with ice water and dried, weighed 16 g. and proved to be practically pure α-hexahydrocinchonane dihydrochloride. The aqueous mother liquor, concentrated to 75 cc., gradually deposited a second crop of crystals, which on recrystallizatiorf from a small volume of water, yielded 7 g. of the characteristic flat needles of the salt of the α-compound. The mother liquors treated in a similar manner finally gave an additional 5 g., or 28 g. in all.

When the aqueous mother liquor from the last fraction was concentrated to about 50 cc. and treated with an equal volume of 40% hydrobromic acid a hydrobromide crystallized rapidly. Under the microscope, it was found to consist mainly of 6-sided elongated plates and prisms of the salt of hexahydrocinchonine, mixed with small, compact prisms of the hydrobromide of β-hexahydrocinchonane, which was easily removed by recrystallization. The yield of the mixed salt was 17 g.

The original alcoholic mother liquor from which the first crude fraction had been obtained was concentrated *in vacuo* to a sirup and dissolved in about 150 cc. of absolute alcohol. On standing in the refrigerator the solution, which had deepened considerably in color, slowly deposited a hard, compact crust of pyramided rhombs which were filtered off after 3 or 4 days and washed with cold absolute alcohol. This fraction weighed 32 g. and consisted mainly of β-hexahydrocinchonane dihydrochloride. The mother liquor from this fraction, concentrated to small bulk and allowed to stand in the refrigerator for several weeks, gave a further slow deposit of crystals, but this fraction was obviously a mixture, and the amount too small to make its study profitable.

α-**Hexahydrocinchonane.**—When the hydrochloride of the α-base first described was dissolved in water and treated with an excess of alkali, a colorless gum was obtained which was easily extracted with ether. The dried extract, on concentration, left an almost colorless sirup which crystallized on standing. When this material was dissolved in sufficient hot ligroin and seeded after it cooled, the base separated as a hard crust of rhombic plates and flat prisms. After repeated recrys-

tallization from ligroin and finally from a small volume of alcohol, and cooling to 0°, the base melted sharply at 106–107° (corr.). Although colorless at first the α-base rapidly turns amber colored on the surface when exposed to sunlight; but when kept in brown glass containers it remains colorless. It is readily soluble in organic solvents, except ligroin which, however, dissolves it appreciably. The base yields stable diacid salts, solutions of which give an olive-green color with ferric chloride and an emerald-green color with permanganate. It couples slowly with diazotized sulfanilic acid to form a red solution of the salt of the azo dye. $[\alpha]_D^{25}$ is 212–217° in absolute alcohol, $c = 1.123$ (g. of substance per 100 cc. of solvent).

Analyses. Calc. for $C_{19}H_{28}N_2$: C, 80.23; H, 9.86; N, 9.86. Found: C, 8010; H, 9.63; N, 1005.

THE DIHYDROCHLORIDE.—This salt is readily purified by recrystallization from water; it forms characteristic long, flat, colorless, glistening needles which darken slightly but do not melt when heated to 285°. It is fairly soluble in water, especially on warming, and very difficultly so in cold absolute alcohol, although the hot solvent dissolves it more readily. Hydrochloric acid or sodium chloride salts it out of a not too dilute aqueous solution. Strong hydrobromic acid likewise precipitates the dihydrobromide as long, flat needles which resemble the hydrochloride. The aqueous solution of the salt is acid to litmus, but not to congo red. $[\alpha]_D^{25}$ is 69.2° in water; $c = 1.026$.

Analyses. Calc. for $C_{19}H_{28}N_2 \cdot 2HCl$: C, 63.83; H, 8.41; N, 7.84; Cl, 19.85. Found: C, 63.60; H, 8.23; N, 8.04; Cl, 19.75.

N-Nitroso-α-hexahydrocinchonane Hydrochloride.—A conc. aqueous solution of the dihydrochloride was treated with a slight excess of a conc. solution of sodium nitrite, yielding a yellow gum which crystallized rapidly when it was rubbed. Since purification of the hydrochloride by simple recrystallization was made difficult because of contamination with the sparingly soluble nitrite of the nitroso compound, the collected salt was dissolved in water and the nitroso base precipitated with alkali and extracted with ether. The washed ether was shaken with a slight excess of dil. hydrochloric acid and the hydrochloride was isolated from the resulting solution by concentration *in vacuo*. Recrystallized from a small volume of water containing a little hyrochloric acid, the hydrochloride separates as flat, lustrous, cream colored needles which melt and effervesce at 203–205°. It is easily soluble in alcohol, quite readily in water, and much less so in dilute hydrochloric acid or salt solution. It gives the Liebermann reaction. $[\alpha]_D^{27}$ is + 68.0° in water; $c = 1.000$. Alkali precipitates the base as a gum which is easily soluble in ether and could not be made to crystallize.

Analyses. Calc. for $C_{19}H_{27}ON_3 \cdot HCl$: N, 12.00; Cl, 10.14. Found: N, 12.15; Cl, 10.13.

N-Acetyl-α-hexahydrocinchonane Hydrochloride.—Two g. of the α-base were dissolved in 10 cc. of benzene, treated with 1 cc. of acetic anhydride and boiled for several minutes, until the benzene boiled away. A small volume of absolute alcohol was added and then alcoholic hydrochloric acid until the solution

was acid to congo red.   On the addition of several volumes of dry ether the hydro-
chloride gradually crystallized.   Reprecipitated with ether from a concentrated
alcoholic solution, the salt formed colorless microscopic leaflets which melted at
235–237° with slight preliminary softening.   It dissolves easily in water or alcohol
and very sparingly in acetone.   Contrary to the unacylated base, it no longer
couples with diazo compounds and gives no color with ferric chloride or permanga-
nate. $[\alpha]_D^{27}$ is $+$ 37.0° in water; $c = 1.000$.   Alkali precipitates the base as a gum
which dissolves easily in ether, but could not make it crystallize.

Analyses.   Calc. for $C_{21}H_{30}ON_2.HCl$: N, 7.73; Cl, 9.78.   Found: N, 7.65; Cl,
9.56.

N-Benzoyl-α-hexahydrocinchonane Hydrochloride.—One and a half g.
of the α-base dissolved in 25 cc. of dry acetone were treated with 1 cc. of benzoyl
chloride.   The solution warmed slightly and deposited a trace of a precipitate.
The filtrate was concentrated to a small volume, acidified with alcoholic hydrochlo-
ric acid, and then treated with dry ether to incipient turbidity.   The crystallization
which ensued on rubbing the vessel was aided by the occasional addition of more
ether.   Recrystallized from methyl ethyl ketone, the salt separated slowly as color-
less microscopic platelets which sinter at about 175° and slowly melt at 215–220°.
The salt is easily soluble in water or alcohol. $[\alpha]_D^{27} = +13.0°$ in water; $c = 0.384$.
The free base was obtained from the salt as a colorless gum which could not be
made to crystallize.

Analyses.   Calc. for $C_{28}H_{32}ON_2.HCl$: N, 6.59; Cl, 8.35.   Found: N, 6.78; Cl,
8.29.

6-Phenylazo-α-hexahydrocinchonane.—Half a gram of aniline dissolved in
15 cc. of N hydrochloric acid was diazotized with 5 cc. of N sodium nitrite and
added to a chilled solution of 1.9 g. of the α-dihydrochloride in 25 cc. of water.
Fifteen cc. of 20% sodium acetate were then added, causing the separation of a
reddish-orange gum, presumably mostly a diazo-amino compound.   An equal
volume of alcohol was added and, when solution was complete, this was followed by
sufficient strong hydrobromic acid to form a deep purple-red solution which
deepened in color on standing.   After the solution had stood for 24 hours at room
temperature, the removal of the alcohol *in vacuo* caused the separation of a deep
red tar which changed when rubbed with water to a purple mass of microscopic
needles.   The separation was aided by the addition of sodium bromide.   The
collected salt was dissolved in warm 50% alcohol and made alkaline with sodium
hydroxide, which caused the purple-red solution to change to a brown-orange color
and deposit the dye base as lustrous orange colored leaflets.   The yield was 0.7
g.   Recrystallized from 85% alcohol it forms rosets of thin, rounded platelets which
melt at 153–156°.   The dye is very readily soluble in benzene and in chloroform
and easily in the other solvents except ligroin.   It dissolves in dil. acids with a deep
red color, purplish-pink in thin layers, while the solution in conc. sulfuric acid
shows a deep brown-red hue which appears light olive-green in thin layers.

Analysis.   Calc. for $C_{25}H_{32}N_4$: N, 14.43; Found: N, 14.65.   .

β-Hexahydrocinchonane.—The free base was obtained from the pure hydrochloride described below as a colorless oil which crystallized on standing. Recrystallized from ligroin, it forms lustrous rhombic plates which, after a final recrystallization from alcohol with the aid of a freezing mixture, melt at 106-106.5°, or at about the same point as the α-base. However, a mixture of both bases melts 15-20° lower. Contrary to the action of the α-base, the β-compound remains colorless on exposure to sunlight. Its salts give the same color reactions as those of the α-base with ferric chloride, with permanganate, and with diazotized sulfanilic acid. It is readily soluble in organic solvents with the exception of ligroin. $[\alpha]_D^{25}$ is 18.15° in absolute alcohol; $c = 0.992$.

Analyses. Calc for $C_{19}H_{28}N_2$: C, 80.23; H, 9.86; N, 9.86. Found: C, 79.90; H, 10.02; N, 9.77.

THE DIHYDROCHLORIDE.—The crude salt (32 g.) previously described as the second fraction (p. 1084) was readily purified by recrystallization from water. It separates slowly as a hard crust of rhombs and pyramidal prisms which occasionally measured 5 mm. in diameter and contain 1 molecule of water of crystallization. When heated rapidly to 230° and then slowly, the salt melts at 237-240° to a liquid filled with bubbles. It is readily soluble in water, appreciably so in alcohol, and insoluble in acetone or in ether. The aqueous solution reacts acid to litmus, but neutral to congo red. $[\alpha]_D^{25}$ is 84.0° in water; $c = 1.000$.

Analyses. Calc. for $C_{19}H_{28}N_2.2HCl.H_2O$: H_2O, 4.80 Found: 4.54. Calc. for $C_{19}H_{28}N_2.2HCl$: N, 7.84; Cl, 19.85. Found: N, 8.07; Cl, 19.68.

Nitroso-β-hexahydrocinchonane and its Hydrochloride.—As in the case of the α-salt the β-hydrochloride yielded the hydrochloride of the nitroso compound, which was purified by recrystallization from water with the addition of hydrochloric acid. The base obtained from the salt formed an amber colored oil which crystallized on standing. When allowed to separate slowly from ligroin, it forms long, prismatic, cream colored needles which melt at 92.5-93.5° (corr.). It is readily soluble in organic solvents and gives the Liebermann reaction. $[\alpha]_D^{25}$ is 107.0° in absolute alcohol; $c = 1.000$.

Analysis. Calc. for $C_{19}H_{27}ON_3$: N, 13.41. Found: N, 13.76.

The salt isolated above forms glistening, cream colored leaflets which contain 1 molecule of water of crystallization and melt with effervescence at 209-211°. It is sparingly soluble in cold water, more readily in alcohol and very sparingly in acetone. $[\alpha]_D^{25}$ is 68.4° in water; $c = 0.746$.

Analyses. Calc. for $C_{19}H_{27}ON_3.HCl.H_2O$: H_2O, 4.90. Found: 4.87. Calc. for $C_{19}H_{27}ON_3.HCl$: N, 12.00; Cl, 10.14. Found: N, 12.36; Cl, 10.28.

N-Benzoyl-β-hexahydrocinchonane Hydrochloride.—One and a half g. of the β-base dissolved in 25 cc. of dry acetone were treated with 1 cc. of benzoyl chloride. After 15 minutes the clear solution was treated with dry ether and rubbed until the salt crystallized. Recrystallized from methyl ethyl ketone, it forms aggregates of colorless needles which melt at 232-234° and are easily soluble in water. $[\alpha]_D^{27}$ is 95.0° in water; $c = 1.000$.

Analyses. Calc. for $C_{26}H_{32}ON_2.HCl$: N, 6.59; Cl, 8.35. Found: N, 6.86; Cl, 8.47.

Hexahydrocinchonine Dihydrobromide.—This salt was obtained as previously described (p. 1084) by the addition of hydrobromic acid to the aqueous mother liquors from the α-dihydrochloride. The crude hydrobromide (17 g.) was recrystallized repeatedly from small volumes of water until the optical rotation became constant. It then formed characteristic long, lustrous, faintly violet, hexagonal plates and prisms which sinter and darken slightly, but do not melt below 290°. The salt is quite readily soluble in water and very sparingly in alcohol. $[\alpha]_D^{27}$ is 50.0° in water; $c = 1.000$. It gives the same color reactions with ferric chloride, with permanganate and with diazotized sulfanilic acid as the salts of the α- and β-bases. Its aqueous solution reacts acid to litmus, but neutral to congo red, and yields a nitrosamine with sodium nitrite. Alkali precipitates the free base as a gum which, so far, has failed to crystallize.

Analyses. Calc. for $C_{19}H_{28}ON_2.2HBr$: C, 49.35; H, 6.55; N, 6.06; Br, 34.63. Found: C, 49.65; H, 6.51; N, 6.34; Br, 34.72.

Reduction of Dihydrocinchonine with Zinc and Hydrochloric Acid.— Twenty g. of dihydrocinchonine were dissolved in 400 cc. of 1:1 hydrochloric acid, and the solution was boiled and treated gradually with 40 g. of granulated zinc (30 mesh). When the zinc was dissolved, water was added to redissolve the pasty double salt which separated as the mixture cooled, and then an excess of ammonia was added. The dried ether extract was concentrated and yielded a yellow oil which was dissolved in 100 cc. of absolute alcohol and treated with dry hydrogen chloride until acid to congo red. When seeded with α-hexahydrocinchonane dihydrochloride, rapid crystallization occurred which was completed in the refrigerator. The dried salt weighed 3 g., or much less in proportion to the yield obtained by the sodium reduction. It proved identical with the salt of the α-base previously isolated. In all probability the β-base is to be found in the mother liquors from the α-hydrochloride, but the poor yield and its solubility rendered its isolation difficult.

B. REDUCTION OF DIHYDROCINCHONANE.

Eighteen g. of dihydrocinchonane hydrochloride (see below) were converted into the base and the dried ether extract concentrated to a sirup. This was dissolved in 320 g. of amyl alcohol and reduced with 24 g. of sodium. After removal of the amyl alcohol, the ether extract yielded a colorless oil which was dissolved in 160 cc. of absolute alcohol and made acid to congo red by dry hydrogen chloride. Thus 10.5 g. of a salt corresponding to that of α-hexahydrocinchonane was obtained. On conversion of this into the free base, an oil was obtained which crystallized readily when seeded with α-hexahydrocinchonane. Recrystallized from ligroin, it formed rhombic plates and prisms which melted at 106–107° (corr.). When mixed with the previously described α-base no change in melting point occurred. $[\alpha]_D^{26}$ is 213.0° in absolute alcohol; $c = 1.000$.

Analysis. Calc. for $C_{19}H_{28}N_2$: N, 9.86. Found: N, 9.98.

When the mother liquor of the α-hydrochloride was concentrated, 6 g. of stout prisms characteristic of the β-dihydrochloride were obtained. The base liberated from this salt melted at 106–106.5° (corr.). No change in melting point was observed when it was mixed with the β-hexahydrocinchonane previously described. $[\alpha]_D^{25}$ is 18.7° in absolute alcohol; $c = 1.016$.

Analysis. Calc. for $C_{19}H_{28}N_2$: N, 9.86. Found: N, 9.94.

From the mother liquors from the above salts none of the characteristic hexagonal plates of the dihydrobromide of hexahydrocinchonine could be separated.

The dihydrocinchonane used above was obtained as follows.

Chloro-dihydrocinchonine and its Hydrochloride.—Sixty-nine g. of dry dihydrocinchonine dihydrochloride were suspended in dry chloroform and poured in a thin stream, with cooling, into a suspension of 115 g. of phosphorous pentachloride in the same solvent. After the mixture had been warmed to 40–50° for several days, it was decomposed with ice and water, and the base liberated from the aqueous extract with alkali and extracted with ether. The oily residue was dissolved in absolute alcohol and neutralized with alcoholic hydrochloric acid. This caused the *hydrochloride* to separate as aggregates of microscopic needles. The mother liquor yielded an additional amount on addition of dry ether, or 43 g. in all. When recrystallized from 85% alcohol it forms radiating aggregates of microscopic needles and narrow leaflets which turn olive colored when heated and melt slowly at 227–228°. It is difficultly soluble in cold water and alcohol, but readily on boiling. $[\alpha]_D^{22}$ is 48.8° in water; $c = 1.107$.

Analyses. Calc. for $C_{19}H_{23}N_2Cl.HCl$: N, 7.98; Cl, 10 09. Found: N, 8.09; Cl, 10.04.

On treating the aqueous solution of the salt with sodium carbonate, an oil formed which crystallized when it was seeded with crystals obtained from a preliminary ethereal extract. Dissolved in acetone and treated with water to incipient turbidity the hydrate of the base separated slowly as rhombs which contained 1.5 molecules of water of crystallization. It softens above 55° and is completely melted to a turbid liquid at 70°. Dried in a desiccator, the hydrate lost its crystalline structure. It is easily soluble in organic solvents. $[\alpha]_D^{24}$ is 36 4° in absolute alcohol; $c = 1.001$.

Analyses. Calc. for $C_{19}H_{23}N_2Cl.1.5H_2O$: H_2O, 7.91; N, 8.20. Found: H_2O, 7.83; N, 8.32.

Dihydrocinchonane and its Hydrochloride.—Thirty-six g. of chlorodihydrocinchonine hydrochloride were reduced as in the case of dihydroquinane.[9] The base was obtained at first as an oil which was dissolved in absolute alcohol and treated with dry hydrogen chloride until it was neutral to moist litmus, the salt separating rapidly as glistening platelets. These were washed with a little absolute alcohol and then with dry acetone, until all the yellow color due to picric acid had been removed. Yield, 22 g. When crystallized from absolute alcohol the hydrochloride forms narrow, glistening plates and prisms which turn yellow and melt

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[9] THIS JOURNAL, 42, 1492 (1920).

slowly at 197–199° to a liquid containing bubbles. It is quite soluble in absolute alcohol, very readily in dry methyl alcohol and chloroform, and difficultly in acetone. $[\alpha]_D^{16}$ is 69.3° in water; $c = 1.068$

Analyses. Calc. for $C_{19}H_{24}N_2.HCl$: N, 8.85; Cl, 11.19. Found: N, 9.04; Cl, 11.38.

On treating a solution of the salt in 50% alcohol with an excess of sodium carbonate and diluting with water, the hydrate of the base crystallized slowly. When dissolved in acetone and treated with water to incipient turbidity, the hydrate separated as rhombs, hexagonal prisms and diamond-shaped platelets which retained 2 molecules of water of crystallization after they had been dried to constant weight in air saturated with water vapor. It is easily soluble in organic solvents and melts at 59.5–60° (corr.). When it is dried in a desiccator or in dry air it loses its crystalline character. $[\alpha]_D^{18}$ is 113.8° in absolute alcohol; $c = 1.014$.

Analyses. Calc. for $C_{19}H_{24}N_2.2H_2O$: H_2O, 11.39; C, 72.10; H, 8.16; N, 8.86. Found: H_2O, 11.17; C, 72.42; H, 8.16; N, 9.17.

C. REDUCTION OF CINCHONINE.

Fifty g. of cinchonine, which had been purified by recrystallization of the sulfate and then of the base from 50% alcohol, were reduced in 1kg. of amyl alcohol with 75 g. of sodium. On concentration, the ethereal extract yielded an amber colored oil which was dissolved in 150 cc. of absolute alcohol and made acid to congo red by the addition of dry hydrogen chloride. On standing in the refrigerator, the almost colorless solution became deep red-brown and deposited colorless needles which were collected and washed with cold absolute alcohol. The yield was 12.5 g.

α-**Tetrahydrocinchonane.**—On converting the above salt as usual into the base an oil was obtained which partially crystallized on standing. When the mixture was dissolved in hot ligroin, cooled, and seeded, a gradual deposition of the base occurred. A second recrystallization from ligroin yielded stout cream colored prisms which melted at 116.5–117.5° (corr.) and were readily soluble in organic solvents, except ligroin. $[\alpha]_D^{18}$ is 209.0° in absolute alcohol, $c = 1.000$, a value practically the same as the specific rotation of α-hexahydrocinchonane. The solution of its hydrochloride gives the same color reactions as the latter with ferric chloride, permanganate and diazotized sulfanilic acid.

Analyses. Calc. for $C_{19}H_{26}N_2$: C, 80.80; H, 9.29; N, 9.93. Found: C, 81.25; H, 9.24; N, 9.90.

To establish its relationship to α-hexahydrocinchonane, the tetrahydro compound was reduced in dil. acetic acid with palladium black and hydrogen. α-Hexahydrocinchonane was isolated from the resulting solution.

Since the products contained in the mother liquor from the crude α-tetrahydrocinchonane dihydrochloride proved too soluble for separation, a clue to the nature of the main products of the reduction was obtained by reduction with palladium

and hydrogen. The mother liquor was accordingly concentrated to remove alcohol and the residue dissolved in 200 cc. of water, a solution of 0.1 g. of palladious chloride added and the mixture reduced with hydrogen. When absorption was complete, the filtrate was concentrated to dryness *in vacuo*, redissolved in absolute alcohol, concentrated again, and the process repeated to remove all water. On dissolving the residue in 200 cc. of absolute alcohol it crystallized rapidly, and yielded 10 g. of hydrochloride which on fractional recrystallization from water gave 3 g. of the characteristic crystals of α-hexahydrocinchonane dihydrochloride. This would indicate that the original separation of the hydrochloride of α-tetrahydrocinchonane from the other products of the reduction of cinchonine had been incomplete.

On treatment with hydrobromic acid the aqueous mother liquor from the α-dihydrochloride yielded the 6-sided platelets of hexahydrocinchonine dihydrobromide.

The alcoholic mother liquor from the above hydrochlorides gave, on concentration, a copious crystallization of β-hexahydrocinchonane dihydrochloride.

D. REDUCTION OF DIHYDROQUININE.

Sixty g. of dihydroquinine, recrystallized from toluene,[10] were reduced as in previous cases with sodium in amyl alcohol. After removal of the latter, an oily base was obtained by extraction with ether. This was dissolved in 200 cc. of absolute alcohol, cooled, and made acid to congo red by passing dry hydrogen chloride through it. A small test portion of the solution was partially precipitated with ether. The gummy precipitate was dissolved in a little absolute alcohol and then ether was added cautiously, causing crystallization to occur gradually. When the main solution was seeded and kept at room temperature (gelatinization occurred in the refrigerator), a copious separation of hair-like needles and denser crystals occurred. After washing with a little absolute alcohol the salt weighed 29 g. The mother liquors from this salt, treated with ether and allowed to stand, yielded a small quantity of fibrous needles which appeared to be the salt of a different base. The quantity of this substance at our disposal and pressure of other work prevented the completion of the study of this fraction and other substances contained in the mother liquors.

Hexahydroquinine Dihydrochloride.—To recrystallize the above first fraction it was dissolved in the minimum amount of boiling methyl alcohol, cooled, and treated with dry ether. The crystals thus formed were recrystallized until the optical rotation had become constant, yielding about 15 g. of optically pure salt. It formed practically colorless rhombic plates which melted at 271–273° with gas evolution and preliminary softening. Although readily soluble in ordinary boiling methyl and ethyl alcohols, the salt is rather sparingly soluble in the dry solvents and almost insoluble in dry acetone and chloroform. It dissolves easily in water, and the solution gives with ferric chloride an emerald-

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[10] In an experiment in which a less pure product was used considerable charring occurred.

green color which changes to an orange color on long standing. $[\alpha]_D^{25.1}$ is $-36.5°$ in water; $c = 1.067$. Sodium bromide salts out the *dihydrobromide* as lustrous plates. Attempts to obtain the free base in crystalline form were unsuccessful.

Analyses. Calc. for $C_{20}H_{30}O_2N_2.2HCl$: C, 59.52; H, 8.00; N, 6.95; Cl, 17.59. Found: C, 59.68; H, 7.92; N, 7.10; Cl, 17.60.

N-Nitroso-hexahydroquinine Hydrochloride.—A solution of the salt in 3 parts of water and a few drops of acetic acid was treated in the cold with a slight excess of sodium nitrite. The hydrochloride soon crystallized as radiating masses of delicate needles which changed on standing to glistening, pale yellow rhombs and prisms which were recrystallized twice from water containing a little hydrochloric acid. It turns lemon-yellow when heated, melts at 212–213° with decompostion, and is fairly soluble in water and alcohol, especially on boiling. It gives the Liebermann reaction and its aqueous solution is not changed at once by permanganate or ferric chloride. $[\alpha]_D^{15}$ is $-85.1°$ in water; $c = 1.034$.

Analyses. Calc. for $C_{20}H_{29}O_3N_3.HCl$: N, 10.62; Cl, 8.96. Found: N, 10.76; Cl, 8.92.

N-Benzoyl-hexahydroquinine.—The dried sirupy base from 4.1 g. of hexahydroquinine dihydrochloride was treated with 15 cc. of benzoyl chloride and heated on the water-bath for 1 hour, after which benzene was added and the solution treated with very dilute hydrochloric acid and ice. A gum separated which gradually dissolved when the mixture was shaken and ether was added to facilitate the process. The aqueous layer was then covered with ether, treated with ice and a slight excess of alkali, and quickly shaken out. The dried ethereal extract left a viscous oil which crystallized after several days. Dissolved in hot ligroin, then cooled and seeded, the base formed lustrous platelets which were purified by solution in a little benzene and addition of ligroin. It melts at 160–160.5° with slight preliminary softening and is soluble in the usual solvents, except ligroin. It does not give a color with ferric chloride. $[\alpha]_D^{20}$ is $-115.2°$ in absolute alcohol; $c = 1.088$.

Analyses. Calc. for $C_{27}H_{34}O_3N_2$: C, 74.61; H, 7.89; N, 6.45. Found: C, 74.81; H, 8.12; N, 6.69.

SUMMARY.

Reduction of dihydrocinchonine with sodium and amyl alcohol yielded a mixture of hexahydrocinchonine and two epimeric hexahydrocinchonanes in the last of which the alcoholic group, as well as the quinoline ring, had suffered reduction. These two bases were also obtained by reduction of dihydrocinchonane. The relationships of these compounds to those obtained by reduction with zinc, and by the reduction of cinchonine were worked out, and necessary intermediates and derivatives described. Dihydroquinine was also reduced, yielding mainly a hexahydroquinine.

SYNTHESES IN THE CINCHONA SERIES.

IX. CERTAIN QUINICINE AND BENZOYLCINCHONA SALTS, CRYSTALLINE ETHYL DIHYDROCUPREINE (OPTOCHIN) BASE, AND OTHER DERIVATIVES.[*]

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In the present communication experiments on a number of miscellaneous cinchona derivatives are assembled, many of which were used as initial material for other work, as will appear in the subsequent papers.

Very few quinicine (quinotoxine) bases or salts have been obtained crystalline up to the present, and recourse has usually been had to the oxalate or some similar salt if a crystalline compound was indeed obtained. In nearly every case investigated by us, however, either the monohydrochloride[1] or the dihydrochloride could be made to crystallize with little difficulty under suitable conditions, and in the exceptions noted the neutral sulfate or hydrobromide crystallized readily. Of these salts, besides dihydroquinicine sulfate, already studied by Hesse,[2] there are described in the present paper N-methyl-quinicine dihydrochloride (I), N-methyl-dihydroquinicine hydrochloride, N-ethyl-quinicine hydrochloride, N-ethyl-dihydroquinicine hydrochloride, N-benzyl-dihydro-quinicine hydrochloride, ethyl-dihydrocupreicine (optotoxin) sulfate, and dihydrocupreicine hydrobromide, a salt of a new quinicine, (II)

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[1] For the hydrochloride of quinicine itself, see THIS JOURNAL, **41**, 832 (1919).

[2] Hesse, *Ann.*, **241**, 273 (1887).

I

II
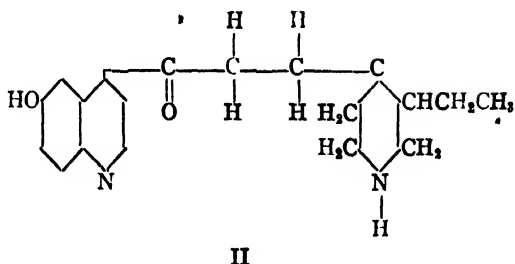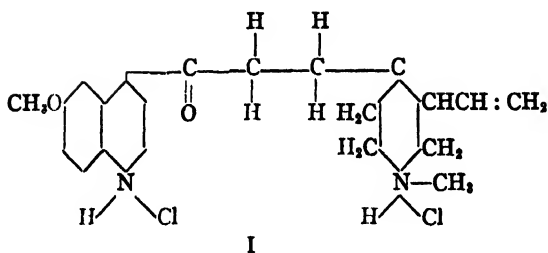
corresponding to dihydrocupreine and dihydrocupreidine.[3] Also in the quinicine series there are described the salts of two glycine derivatives in which the NH group of the piperidine nucleus has been condensed with chloro-acetyl derivatives of aromatic amines.

We have also described benzoyl-cinchonidine dihydrochloride benzoyl-dihydrocinchonidine hydrochloride, benzoyl-quinine dihydrochloride, and benzoyl-dihydroquinine hydrochloride which, as far as we know, are new.   Cinchotenine methyl ester,
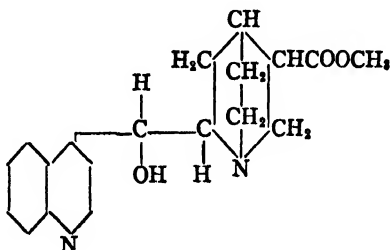
cinchotenine ethyl ester hydrochloride, and cupretenine (quitenol) methyl ester dihydrochloride, we also believe have not been described.

Hitherto only crystalline salts of ethyl dihydrocupreine (optochin) have been prepared,[4] and Giemsa and Halberkann report their inability

[3] THIS JOURNAL, **41**, 821, 827 (1919).
[4] *Ibid.*, **41**, 824 (1919). Giemsa and Halberkann, *Ber.*, **51**, 1332 (1918).

to crystallize the base. It was found, however, that after the initial crystals had been obtained by spontaneous evaporation of a toluene solution, the base could readily be obtained crystalline by seeding concentrated solutions in this solvent, separating as irregular leaflets containing toluene of crystallization, at least a part of which is retained on air drying.

Finally, the ethyl bromides of dihydroquinine and ethyl-dihydrocupreine are described, as well as the dihydrobromide of hydrobromocupreine (or hydrobromo-apoquinine), and existing data on hydrobromocinchonidine are completed.

<div align="center">

EXPERIMENTAL.[5]

A. QUINICINE SALTS.

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Dihydroquinicine Sulfate.[6]—Dihydroquinicine was prepared from dihydroquinine[7] sulfate according to v. Miller, Rohde and Fussenegger[8] and the crude, oily base neutralized to wet litmus in absolute alcohol with 30% aqueous sulfuric acid and treated with dry ether until the initial turbidity just redissolved. The salt separated on rubbing, and was recrystallized by dissolving in hot 95% alcohol, cooling, adding an equal volume of acetone, seeding and letting stand in the cold. It has the properties mentioned by Hesse, and in addition, comes to equilibrium in the air with 3 molecules of water of crystallization (or its equivalent). When anhydrous, it gives $[\alpha]_D^{21.4}$ −8.3° in water, $c = 0.968$, and softens and turns yellow at 173°, melting at 174–176° to a brown liquid. The dried salt dissolves readily in absolute methyl or ethyl alcohol, separating from the latter on rubbing, presumably with alcohol of crystallization.

Analyses. Calc. for $(C_{20}H_{26}O_2N_2)_2.H_2SO_4.3H_2O : H_2O$, 6.72. Found : 6.67. Calc. for $(C_{20}H_{26}O_2N_2)_2.H_2SO_4$: N, 7.47; SO$_4$, 12.79. Found: N, 7.47, SO$_4$, 12.68.

N-Methylquinicine Dihydrochloride.—Quinidine methiodide was decomposed according to the method Freund and Rosenstein[9] used in the case of cinchonine methiodide, namely, heating in an autoclave with an excess of strong alkali. According to the literature the salts are oily, but a test portion eventually crystallized when treated with an excess of conc. hydrochloric acid, diluted with dry acetone, and allowed to evaporate spontaneously, with replacement of the acetone from time to time and rubbing. The base was accordingly dissolved in a little absolute alcohol, acidified to wet congo red paper with conc. hydrochloric acid,

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[5] Melting points and optical rotations were determined as in previous papers.

[6] Not fully described by Hesse, Ref. 3.

[7] THIS JOURNAL, **41**, 819 (1919). Ger. pat. 252, 136.

[8] v. Miller, Rohde and Fussenegger, *Ber.*, **33**, 3228 (1900).

[9] Freund and Rosenstein, *Ann.*, **277**, 279 (1893).

diluted with dry acetone until the initial turbidity just redissolved, and seeded. Crystallization of the salt as rhombic aggregates was completed by rubbing and adding more dry acetone from time to time. The dihydrochloride was recrystallized by a similar process. The air-dry salt dissolves readily in water with a bright yellow color, appearing greenish in thin layers and turning more greenish on dilution. It has a definite anesthetic effect on the tip of the tongue. Dried *in vacuo* at room temperature over sulfuric acid it gives $[\alpha]_D^{25}$ +16.6° in water, $c = 0.992$, and softens at 140–150° to a jelly, melting at 153–155° to give a yellow liquid containing bubbles. The substance gives the thallcoquinine test. The base obtained from the purified salt did not crystallize.

Analyses. Calc. for $C_{21}H_{26}O_2N_2.2HCl.H_2O$: H_2O, 4.20. Found: 4.63. Calc. for $C_{21}H_{26}O_2N_2.2HCl$: N, 6.82; Cl, 17.24. Found: N, 6.91; Cl, 16.89.

N-Methyl-dihydroquinicine Hydrochloride.—Sixty g. of dihydroquinine methiodide were heated for 4 hours at 160–165° in an autoclave with 300 cc. of water and 22 g. of sodium hydroxide.[9] The oil obtained from the ether extract was taken up in absolute alcohol. After neutralizing with absolute alcoholic hydrochloric acid the solution was concentrated *in vacuo*, dissolved in the minimum amount of dry acetone, and seeded with crystals obtained from a similarly treated test portion. Concentration of the mother liquor and addition of ether yielded a second crop, a total of 25 g. being obtained. Recrystallized from absolute alcohol by the addition of dry ether, the hydrochloride separates as cream colored microscopic prisms and needles with wedge-shaped ends. It dissolves in water to form a solution with a dull yellow color, which changes to the characteristic bright yellow-green of the quinicine di-acid salts on adding dil. hydrochloric acid. An aqueous solution has a slowly developing, definite anesthetic effect on the tip of the tongue. The air-dry salt softens to a jelly at about 120° and melts completely at about 145°. The anhydrous compound, when rapidly heated to 150°, then slowly, melts to a jelly at 150–153° and is completely fluid at 163°. It dissolves readily in absolute alcohol, dry acetone, or chloroform, and gives $[\alpha]_D^{25.4}$ −9.4° in water; $c = 1.015$.

Analyses. Calc. for $C_{21}H_{28}O_2N_2.HCl.0.5H_2O$: H_2O, 2.34. Found: 2.93. Calc. for $C_{21}H_{28}O_2N_2.HCl$: N, 7.44; Cl, 9.41. Found: N, 7.46; Cl, 9.27.

The base obtained from the purified salt failed to crystallize.

N-Ethyl-quinicine Hydrochloride.—The oily base obtained from quinidine ethyl bromide was converted into the salt as in the preceding cases. Recrystallized twice from 95% alcohol, it separates as faintly yellow, short rods which are anhydrous. It is quite soluble in dry methyl alcohol and slowly but freely soluble in water, the aqueous solution being weakly bitter and having definite anesthetic properties. It gives a dark blue-gray thalleoquinine test, changing to lilac. When rapidly heated to 200°, then slowly, it softens at 201°, and melts at 202–204° to a dark liquid which slowly decomposes. $[\alpha]_D^{25}$ is + 68.1° in water, $c = 0.665$, a much higher value than that obtained in the case of other closely related salts.

Analyses. Calc. for $C_{22}H_{28}O_2N_2.HCl$: N, 7.21; Cl, 9.12. Found: N, 7.33; Cl, 9.02.

N-Ethyl-dihydroquinicine Hydrochloride.—This salt, obtained from dihydroquinine ethyl bromide (see below), was recrystallized from absolute alcohol and forms rosets of minute platelets which, when rapidly heated to 195° then slowly, soften to a dark tar at 196–198° and melt completely at 202°. It dissolves readily in dry methyl alcohol or chloroform, less readily in absolute alcohol, and sparingly in boiling dry acetone. $[\alpha]_D^{17}$ is $-14.4°$ in water; $c = 1.007$. It also has anesthetic properties.

Analyses. Calc. for $C_{22}H_{30}O_2N_2.HCl$: N, 7.17; Cl, 9.07. Found: N, 7.88; Cl, 8.98.

N-Benzyl-dihydroquinicine Hydrochloride.[10]—The crude base was taken up in dry acetone, neutralized with absolute alcoholic hydrochloric acid, and seeded with crystals obtained by evaporating a neutral alcoholic solution, adding dry acetone, and letting stand. The yield was 10.1 g. Recrystallized from absolute alcohol with the aid of dry ether the salt forms rosets of long, narrow platelets. It dissolves readily in methyl alcohol or chloroform, less easily in cold absolute alcohol, and turns gummy under a little water, dissolving on dilution without color, and turning pale yellow on adding hydrochloric acid. When rapidly heated to 160°, then slowly, it melts with slight preliminary softening at 161–164°, with slow decomposition. $[\alpha]_D^{24.5}$ in 50% alcohol is $-65.9°$; $c = 1.093$. It also has anesthetic properties.

Analyses. Calc. for $C_{27}H_{32}O_2N_2.HCl$: N, 6.19; Cl, 7.83. Found: N, 6.23; Cl, 7.71.

Ethyl-dihydrocupreicine (Optotoxine) Sulfate.—Although the quinicine prepared from ethyl-dihydrocupreine (optochin) is mentioned by Morgenroth[11] we have been unable to find any description of the base or its salts. The sulfate was found to crystallize readily, and was prepared as follows: 50 g. of ethyl-dihydrocupreine (Zimmer) were dissolved in 600 cc. of water and 100 cc. of 50% acetic acid and boiled in an oil-bath for 30–35 hours.[12] The free base obtained from the brown-orange solution was taken up in absolute alcohol and made very slightly acid with conc. sulfuric acid. The sulfate separated slowly when left in the cold and after rubbing. The filtrate yielded more when treated with dry acetone and ether. The yield was 28 g. Recrystallized from absolute alcohol it forms voluminous, hair-like needles which dissolve in water to form a solution with a pale greenish-yellow color changing to an intense lemon-yellow with a little mineral acid. The anhydrous salt melts at 164–166° to give a yellow liquid, has $[\alpha]_D^{25}$ $-7.8°$ in water $c = 1.090$, dissolves readily in methyl alcohol, rather sparingly in cold absolute alcohol, and gelatinizes under dry chloroform, dissolving with difficulty. It acts as an anesthetic on the tip of the tongue.

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[10] THIS JOURNAL, 41, 2102 (1919).

[11] Morgenroth, cf. *C. A.*, 13, 2207 (1919).

[12] Cf. v. Miller and Rohde, *Ber.*, 28, 1064 (1895).

Analyses. Calc. for 2.5 H_2O: 5.47. Calc. for 1 C_2H_5OH: 5.59. Found: 5.81. Calc. for $(C_{21}H_{26}O_2N_2)_2.H_2SO_4$: N, 7.20; $SO_4^=$, 12.33. Found: N, 7.41; $SO_4^=$, 12.34.

Dihydrocupreicine Hydrobromide.—Twenty-five g. of crude, oily dihydro-quinicine[18] were demethylated with aqueous hydrobromic acid (sp. gr. 1.49).[14] The solution was concentrated to dryness *in vacuo*, taken up in a little hot water, cooled, and 10% aqueous sodium hydroxide was cautiously added until considerable precipitate formed but the solution still remained faintly acid to litmus. The collected salt was recrystallized from water, separating as olive-yellow aggregates of pointed platelets. The yield was 12.5 g. The hydrobromide melts slowly, with slight preliminary softening, at 213–215° to a brown liquid which gradually blackens. It dissolves in water to form a solution with a yellow color, which becomes more intense on further addition of acid. A dilute aqueous solution gives a deep olive-brown color with ferric chloride and, when made alkaline, couples readily with diazotized sulfanilic acid. It dissolves with difficulty in cold absolute alcohol, more easily on boiling, and is quite soluble in cold dry methyl alcohol. A concentrated aqueous solution gives an orange colored precipitate of the base with ammonia, but the base could not be made to crystallize. $[\alpha]_D^{21.5}$ in water is $-5.4°$; $c = 0.827$.

Analyses. Calc. for $C_{19}H_{24}O_2N_2.HBr$: N, 7.13; Br, 20.32. Found: N, 7.31; Br, 20.11.

B.   GLYCINE DERIVATIVES OF QUINICINE.

Quinicylglycin-anilide Dihydrochloride, $R:N.CH_2CONHC_6H_5.2HCl$.—Four g. of quinicine oxalate, 1.7 g. of chloro-acetanilide, 2 g. of sodium iodide, 4 g. of crystalline sodium acetate, 25 cc. of alcohol, 10 cc. of N sodium hydroxide solution, and 15 cc. of water were boiled on the water-bath for 3 hours, with a little more alcohol to hold in solution the brown oil which soon began to separate. An additional 10 cc. of N sodium hydroxide was finally added and the mixture diluted with hot water, precipitating a greenish gum which resisted all efforts at crystallization. After washing with water it was treated with about 25 cc. of 1:1 hydrochloric acid. The dihydrochloride which crystallized was taken up in hot water, the solution boiled with bone black to remove impurities, and the filtrate chilled and treated with hydrogen chloride until just turbid. The salt separated as sheaves and rosets of delicate, pale yellow needles. Crystallization was completed by passing in more hydrogen chloride, the total yield being 2.3 g. The substance is rather sparingly soluble in cold water, more easily on warming, with a yellow color, and when dry gradually sinters to a jelly above 130°, melting and evolving gas at about 190°.

Analyses. Calc. for $C_{28}H_{31}O_3N_3.2HCl$: N, 7.93; Cl, 13.37. Found: N, 8.16; Cl, 13.95.

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[18] P. 215.

[14] Cf. THIS JOURNAL, **41**, 821 (1919).

Quinicylglycine-*p*-hydroxyanilide Acid Sulfate.—This substance was prepared as in the case of the preceding compound, using *p*-chloro-acetylamino-phenol.[15] As neither the base nor the dihydrochloride crystallized, the crude product was rubbed with 25% sulfuric acid, when it soon became crystalline. Recrystallized from 50% alcohol containing a drop of dil. sulfuric acid, it formed rosets of orange colored leaflets and needles which were dried *in vacuo*. It melts at 212–215° with preliminary darkening and softening, and is very difficultly soluble in cold water but dissolves on boiling. It is also sparingly soluble in absolute alcohol or dry methyl alcohol, and dissolves in dil. sodium hydroxide solution with the formation of a pale yellow color.

Analyses. Calc. for $C_{28}H_{31}O_4N_3.H_2SO_4$: N, 7.36; SO_4^-, 16.82. Found: N, 7.28; SO_4^-, 17.12.

C. HYDROCHLORIDES OF CERTAIN BENZOYLATED CINCHONA ALKALOIDS.

Benzoyl-cinchonidine Dihydrochloride.—Thirty g. of powdered cinchoni-dine were added in small portions to 60 g. of benzoyl chloride on the water-bath, with stirring.[16] The salt suddenly separated before all of the alkaloid had gone into solution and heating was continued for 45 minutes. An equal volume of dry acetone was added and the mixture boiled under a reflux condenser until the lumps had hardened, after which they were ground up in a mortar and again boiled for several hours. The yield was 33.8 g. Recrystallized from absolute alcohol by the addition of dry ether, the salt formed rosets of club-shaped, prismatic needles. The anhydrous salt darkens and sinters above 200°, melting and decomposing at 208–211°, it dissolves readily in water or methyl alcohol, less easily in dry chloro-form, and sparingly in cold absolute alcohol but readily on warming.

Analyses. Calc. for $C_{26}H_{26}O_2N_2.2HCl.H_2O$: H_2O, 3.68. Found: 3.05. Calc. for $C_{26}H_{26}O_2N_2.2HCl$: N, 5.95; Cl, 15.04. Found: N, 5.99; Cl, 14.77.

Benzoyl-dihydrocinchonidine Hydrochloride.—When 19.7 g. of benzoyl-cinchonidine dihydrochloride were dissolved in 150 cc. of water, treated with 5 cc. of 2% palladious chloride solution, and shaken with hydrogen, the calculated amount of gas was absorbed. The filtered solution was diluted and the base precipi-tated with ammonia, 15.4 g. of amorphous product being obtained. A solution of the base in absolute alcohol was neutralized with absolute alcoholic hydrochloric acid and treated with dry ether. The salt thus obtained was recrystallized by a similar process and formed rhombic crystals which were air-dried. It is rather sparingly soluble in water, dissolves slowly but freely in absolute alcohol or dry acetone, and is easily soluble in dry chloroform. The anhydrous salt gives $[\alpha]_D^{17.8}$ +124.9° in absolute alcohol, $c = 1.093$, and softens to a jelly at 160–165°, gradually melting at 185–190° to form a yellow liquid filled with bubbles.

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[15] THIS JOURNAL, **39**, 1442 (1917).

[16] Cf. Wunsch, *Compt. rend.*, **119**, 407 (1894).

Analyses. Calc. for $C_{26}H_{28}O_2N_2.HCl.H_2O$: H_2O, 3.96. Found: 3.74. Calc. for $C_{26}H_{28}O_2N_2.HCl$: N, 6.42; Cl, 8.12. Found: N, 6.65; Cl, 8.19.

Benzoylquinine Dihydrochloride.—Anhydrous quinine when treated 45 minutes with benzoyl chloride yielded a crystalline salt.[17] This was recrystallized as in the preceding case, forming short, transparent prisms which were warmed to 60° for a few moments, evacuated and air-dried. It dissolves readily in water, methyl alcohol or chloroform, and somewhat less easily in absolute alcohol. The anhydrous salt gives $[\alpha]_D^{23} + 88.7°$ in water, $c = 0.892$, and when rapidly heated to 225°, then slowly, turns yellow and softens, finally melting and decomposing at 229–232°.

Analyses. Calc. for $C_{27}H_{28}O_3N_2.2HCl.H_2O$: H_2O, 3.59. Found: 4.21. Calc. for $C_{27}H_{28}O_3N_2.2HCl$: N, 5.59; Cl, 14.15. Found: N, 5.95; Cl, 13.96.

Benzoyl-dihydroquinine Hydrochloride.—Benzoylquinine dihydrochloride was reduced in the same way as the cinchonidine analog, and the crude, amorphous base was converted into the hydrochloride as in the case of the dihydrocinchonidine analog. The solution was concentrated to dryness *in vacuo* and the residue was dissolved in dry acetone, treated with dry ether, and then with ligroin until slightly turbid. The salt gradually crystallized and was purified by a repetition of the process, separating as flat, cream colored prisms which dissolve sparingly in cold water. The anhydrous salt melts and decomposes at 235–240° with preliminary softening and gives $[\alpha]_D^{24} + 140.6°$ in absolute alcohol; $c = 1.298$.

Analyses. Calc. for $C_{27}H_{30}O_3N_2.HCl.0.5H_2O$: H_2O, 1.89. Found: 1.92. Calc. for $C_{27}H_{30}O_3N_2.HCl$: N, 6.00; Cl, 7.60. Found: N, 6.10; Cl, 7.55.

TENINE ESTER DERIVATIVES.

Cinchotenine Methyl Ester.—Anhydrous cinchotenine was esterified in dry methyl alcoholic solution by saturation with dry hydrogen chloride. After removing the alcohol the ester was obtained from an aqueous solution of the residue by cautiously adding sodium carbonate solution. Recrystallized from alcohol it forms glistening prismatic plates which dissolve rather sparingly in the cold in the usual neutral solvents. When rapidly heated to 240°, then slowly, the ester darkens and sinters above this point, melting and decomposing at 243–244.5°. $[\alpha]_D^{23}$ in methyl alcohol is $+118.7°$; $c = 0.206$.

Analyses. Calc. for $C_{19}H_{22}O_3N_2$: N, 8.59. Found: N, 8.70.

Cinchotenine Ethyl Ester Hydrochloride.—This salt was prepared by neutralizing a solution of the ester base in absolute alcohol, and treating with dry ether. The salt separated as aggregates of minute plates which decompose at about 250°.

Analyses. Calc. for $C_{20}H_{24}O_3N_2.HCl.0.5H_2O$: H_2O, 2.34. Found: 2.31. Calc. for $C_{20}H_{24}O_3N_2.HCl$: Cl. 9.41. Found: 9.70.

Cupretenine (Quitenol)[17] Methyl Ester Dihydrochloride.—The ester was

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[17] Quitenine was demethylated by boiling with hydrobromic acid (sp. gr. 1.49) instead of with hydriodic acid, as given by Bucher, *Monatsh.*, **14**, 603 (1893).

prepared and isolated as in the case of the cinchotenine analog. The dried, amorphous ester was converted into the dihydrochloride by means of an excess of absolute alcoholic hydrochloric acid and the solution treated with dry ether. Recrystallized in the same way it formed rosets of silky needles which gradually softened and turned yellow when heated, melting to a paste and evolving gas at about 200°. It is readily soluble in methyl alcohol and in water, the aqueous solution coupling with diazotized sulfanilic acid when made alkaline.

Analyses. Calc. for $C_{19}H_{24}O_4N_2.2HCl$: N, 6.51; Cl, 17.07. Found: N, 6.70; Cl, 16.30.

CRYSTALLINE ETHYL-DIHYDROCUPREINE (OPTOCHIN) AND OTHER SIMPLE CINCHONA DERIVATIVES.

Ethyl-dihydrocupreine (Optochin).—Led by the ease with which we found dihydroquinine to crystallize from toluene we attempted to obtain ethyl-dihydrocupreine crystals from this solvent. The base dissolved easily, and on allowing the solution to evaporate spontaneously, crystals soon formed on the walls of the vessel. Five g. of the amorphous base (Zimmer) were accordingly dissolved in about 10 cc. of hot toluene, cooled, and seeded, the base gradually crystallizing as aggregates of irregular platelets which were again recrystallized from toluene, additional amounts being obtained from the mother liquors on adding ligroin and seeding. The crystals retained toluene of crystallization even on air drying, but as the combustions obtained on such products indicated that water was present as well, we are unable to say how much toluene was retained. The air-dry substance gives $[\alpha]_D^{25.6}$ $-112.7°$ in absolute alcohol, $c = 1.002$, melts at 80–4° with preliminary softening, and is less soluble in benzene, toluene, or ligroin than in the other usual organic solvents. After removing the toluene and water *in vacuo* first at room temperature, then at 100°, the residue melted at 123–128° with preliminary softening and gave $[\alpha]_D^{25}$ $-136.2°$ in absolute alcohol, $c = 1.005$, figures comparable with those given by the best commercial specimens of the amorphous base.

Analyses. Subs., air-dry, 0.3254: loss, 0.0510, or 15.67%; 15.66 on another preparation. Calc. for $C_{21}H_{28}O_2N_2$: C, 74.07; H, 8.29. Found: C, 74.31; H, 8.17.

Ethyl-dihydrocupreine Ethyl Bromide.—Two g. of the base and a slight excess of ethyl bromide were boiled in dry acetone for 4 hours, and the solvent then boiled off. The residue gradually crystallized on standing, as a radiating fibrous mass. This was recrystallized by dissolving in boiling dry acetone and adding dry ether. After further recrystallization it forms rosets of minute, rhombic plates which dissolve quite readily in water, especially on warming. The anhydrous bromide gives $[\alpha]_D^{25}$ $-111.8°$ in water, $c = 1.100$, softens to a viscous mass above 120° and at 185° yields a completely fluid, yellow mass containing bubbles. It dissolves readily in methyl or ethyl alcohol, acetone, or chloroform.

Analyses. Calc. for $C_{23}H_{33}O_2N_2Br.H_2O$: H_2O, 3.86. Found: 3.89. Calc. for $C_{23}H_{33}O_2N_2Br$: Br, 17.78. Found: 1772.

Dihydroquinine Ethyl Bromide.—The components were boiled for 4 hours in a mixture of equal volumes of dry chloroform and dry acetone. Washed with acetone and recrystallized first from water and then from alcohol by adding ether, the salt formed rosets of glistening platelets. When anhydrous it gives $[\alpha]_D^{28,5}$ $-111.1°$ in water $c = 1.004$, and when rapidly heated to 185°, then slowly, melts slowly at 188–190° with slight decomposition. It is less soluble in acetone than the optochin derivative.

Analyses. Calc. for $C_{22}H_{31}O_2N_2Br.0.5H_2O : H_2O, 2.03.$ Found: 2.06. Calc. for $C_{22}H_{31}O_2N_2Br$: Br, 18.36. Found. 18.31.

Hydrobromocinchonidine.—This substance was prepared as in the case of the hydrobromo compound described in the next paragraph, and to Léger's[18] description we can add only the following data. When rapidly heated to 175°, then slowly, the base discolors somewhat and melts to form a reddish liquid, with decomposition at 176–177°. $[\alpha]_D^{n,5}$ is $-226.8°$ in dry methyl alcohol; $c = 0.1608.$

Analyses. Calc. for $C_{19}H_{23}ON_2Br$: N, 7.47. Found: N, 7.52.

Hydrobromocupreine (or Hydrobromo-apoquinine) Dihydrobromide.—Ten and a half g. of quinine were heated for 6 hours at 110° in an oil-bath with 60 cc. of hydrobromic acid (sp. gr. 1.49). On cooling and standing for several days the above salt crystallized. Recrystallized from water containing a little hydrobromic acid, the dihydrobromide separated as delicate, tawny, voluminous needles. The yield was 4.5 g. Like solutions of the di-acid dihydrocupreine salts, the very faintly yellow solution in water turns a deeper yellow on neutralization (the reverse of the phenomena observed in the case of the quinicines), and then gives a pale brown color with ferric chloride. The base is precipitated by sodium carbonate, redissolving on adding sodium hydroxide to yield a solution which couples with diazotized sulfanilic acid. The anhydrous salt gives $[\alpha]_D^{21}$ $-161.8°$ in water, $c = 1.022$, and softens slightly when heated, sintering at 190–195° and slowly intumescing at 197–203°. It is somewhat soluble in cold absolute alcohol, the solution cystallizing on rubbing.

Analyses. Calc. for $C_{19}H_{23}O_2N_2Br.2HBr.3.5H_2O : H_2O$, 10.23. Found: 10.24. Calc. for $C_{19}H_{23}O_2N_2Br.2HBr$: Br^-, 28.91. Found: 29.20.

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[18] Léger, *Bull. soc chim.*, [4] **25**, 572 (1919).

SYNTHESES IN THE CINCHONA SERIES.

X. DIHYDROCINCHONICINOL AND THE DIHYDROQUINICINOLS.[*]

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(Received for publication, July 28, 1921.)

In the course of the bacteriological study of certain cinchona deriva-
tives undertaken in conjunction with Dr. Martha Wollstein and Dr.
Lloyd D. Felton it developed that in general the dihydrogenated
alkaloids of the quinicine type (I) were less pneumococcidal than the
original alkaloids (II) from which they were derived. While it seemed
possible that this effect was in some way due to the breaking up of
the fused quinuclidene nucleus into the simpler piperidine ring, it
seemed also possible that the loss of the secondary hydroxyl group
might play a part, especially as dihydroquinane and its related
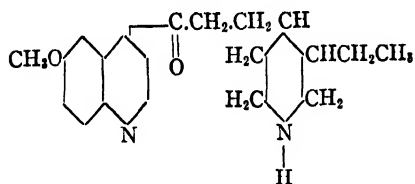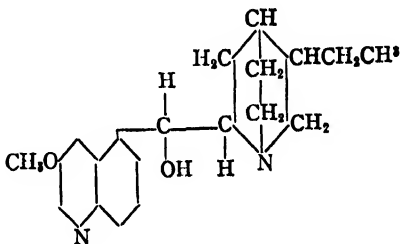
alkaloids,[1] (III) in which the quinuclidene nucleus is still intact, had
previously been found to be less active than the parent alkaloids. It
was therefore hoped that the restoration of the secondary alcohol group
in the quinicine series would enhance the pneumococcidal power of
these alkaloids. While it was found possible to reduce the keto group

[*] Presented at the Annual Meeting of the American Chemical Society, New
York, September, 1921.
[1] THIS JOURNAL, **42**, 1489 (1920).

in the desired sense, the resulting alkaloids (IV) were devoid of marked pneumococcidal power, even when the tertiary character of the piperidine nitrogen atom was restored by methylation or ethylation.

III   IV
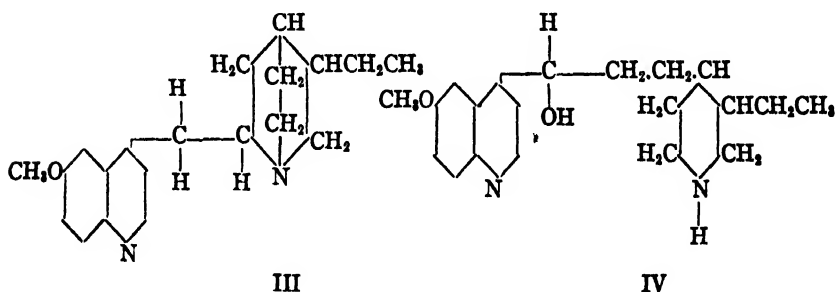
The reduction was accomplished with the aid of palladium black and hydrogen, and we have named the new alkaloids so prepared the dihydrocinchonicinols and dihydroquinicinols, depending on whether the alkoxy group is absent or present.   It is obvious that in the reduction of the keto group the central carbon atom is restored to its original condition of asymmetry, so that optical isomers should result.   The reduction products proved, as expected, to be mixtures and, depending on the particular case, could be separated into the optical isomers either as the free base or as an appropriate salt.   In every case the *d*-compound proved the easier to isolate, and of those investigated, only in the case of N-methyl-dihydroquinicinol, could both the *d*- and *l*-bases be obtained crystalline.   Dihydrocinchonicinol was separated into the *d*- and *l*- sulfates; *d*-dihydroquinicinol crystallized, but the *l*-isomer could be isolated only as the dihydrochloride; *d*-N-ethyl-dihydroquincinol and *d*-O-ethyl-dihydrocupreicinol (V) (corresponding to ethyl-dihydrocupreine, or optochin), were obtained crystalline, and while in the former case a second dihydrochloride was obtained, no crystalline derivative of *l*-O-ethyl-dihydrocupreicine could be prepared.   The mixture of N-benzyl-dihydroquinicinols also failed to yield crystalline derivatives.

   d-N-Methyl-dihydroquinicinol   and   *d*-N-ethyl-dihydroquinicinol were also prepared by alkylation of the parent *d*-dihydroquinicinol, and similar derivatives were also formed from *d*-O-ethyl-dihydrocupreicinol crystallizing with little difficulty.

V

An interesting consequence of the restoration of the secondary hydroxyl group in the case of the dihydroquinicinols (and O-ethyl-dihydrocupreicinol) was the restoration of the bluish fluorescence of the bases when dissolved in excess nitric or sulfuric acid, thus support-ing Kaufmann's statement that the alkoxy group and the secondary hydroxyl are necessary to produce this phenomenon.[2]   The dilute acid solutions were also relatively stable to potassium permanganate.

It is hoped soon to study the pharmacological properties of this new series of alkaloids.

EXPERIMENTAL.

d- and *l-*Dihydrocinchonicinol Sulfates.[3]—Fifty g. of air-dry cinchonicine oxalate were dissolved in water and dil. hydrochloric acid, the solution made alkaline with sodium hydroxide, shaken out with ether, and the extract dried for a few moments and concentrated.   The residue was taken up in about 100 cc. of water and 14 g. of conc. sulfuric acid and shaken in an atmosphere of hydrogen with the palladium black from 8 cc. of 2% palladious chloride solution, with the addition of 3 cc. of the palladium chloride solution after reduction had commenced, in order to accelerate the reaction.   The absorption was 5570 cc. (Calc. for $2H_2$, 5965 cc.). In a later experiment cinchonicine oxalate itself was reduced after dissolving with the aid of an equivalent of sulfuric acid and the absorption found to be slightly greater than that calculated.   The solution was diluted, decanted from the palla-dium, made alkaline with sodium hydroxide, and the base extracted with ether. The ether extract was thoroughly dried, finally over sodium hydroxide, concen-trated, dissolved in 150 cc. of absolute alcohol and made just barely acid to litmus with conc. sulfuric acid.   On seeding with crystals obtained from a portion which was precipitated with dry acetone and allowed to stand for several days, 7.6 g. of

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[2] Kaufmann, *Ber.*, **46**, 1827 (1913).
[3] After most of the work on the quinicinols had been completed it was found that Kaufmann and Huber [*Ber.*, **46**, 2919 (1913)] mention that cinchonicine absorbs 4 atoms of hydrogen, giving yellowish, oily "hydrocinchotoxol" which was not investigated.

the crude d-sulfate gradually crystallized. This was recrystallized first from absolute alcohol, then boiled out with a little absolute alcohol, and finally dissolved in the minimum amount of 95% alcohol. Dry acetone was then added to marked turbidity, followed by treatment with bone black and filtering, and again the addition of dry acetone to incipient turbidity. The rotation of the salt which then crystallized was found to have attained a constant value.

d-Dihydrocinchonicinol sulfate forms cream colored, microscopic needles which dissolve readily in water to form a solution with a pale yellow color and are difficultly soluble in absolute alcohol, but somewhat mòre so in dry methyl alcohol. The anhydrous salt sinters and turns brown at 222°, melting at 223–224° with slow gas evolution, and gives $[\alpha]_D^{18} + 63.6°$ in water; $c = 1.014$.

Analyses. Calc. for $(C_{19}H_{26}ON_2)_2.H_2SO_4.2H_2O$: H_2O, 4.93. Found: 5.23. Calc. for $(C_{19}H_{26}ON_2)_2.H_2SO_4$: C, 65.65; H, 7.84; N, 8.07; $SO^-{}_4$, 13.83. Found: C, 65.35; H, 7.47; N, 8.27; $SO^-{}_4$, 13.98.

The filtrate from the crude d-sulfate was treated with dry acetone and stirred until the initial gummy precipitate dissolved with difficulty, the solution bottled, and allowed to stand in the ice-box for about 2 weeks. The drab-colored precipitate, which resembled the first fraction, weighed 9.1 g., but after recrystallization from absolute alcohol only 2.2 g. were recovered, and the product was strongly levorotatory. It was accordingly boiled with a little absolute alcohol, cooled, the colored alcoholic solution poured off, and the residue dissolved in boiling dry methyl alcohol. Dry acetone was then added to incipient turbidity, the solution treated with bone black and rapidly filtered; more dry acetone was added to incipient turbidity and the mixture allowed to stand. The l-sulfate separated as rosets of minute, cream colored leaflets which were dried *in vacuo* over sulfuric acid. $[\alpha]_D^{18}$ in water was constant at $-57.3°$; $c = 1.109$. When rapidly heated to 230°, then slowly, the salt melts with preliminary softening at 232–234°, with gas evolution. In properties, as well as in appearance, it greatly resembles its optical isomer.

Analyses. Calc. for $(C_{19}H_{26}ON_2)_2.H_2SO_4$: C, 65.65; H, 7.84; N, 8.07; $SO^-{}_4$, 13.83. Found: C, 65.74; H, 7.87; N, 8.14; $SO^-{}_4$, 14.03.

Neither the d-nor l-base, liberated from its sulfate, could be made to crystallize.

On addition of more dry acetone to the mother liquors from the d- and l- sulfates mixtures of the d-and l- forms were obtained.

DIHYDROQUINICINOL

d-Dihydroquinicinol Nitrate.—Sixty g. of recrystallized quinicine hydrochloride[4] were dissolved in 180 cc. of water and 7.5 cc. of conc. sulfuric acid and reduced with palladium and hydrogen. The absorption was 7655 cc. (Calc. for 2 H_2, 7453 cc.) The filtrate from the palladium was neutralized to litmus with 10% aqueous sodium hydroxide, and care was taken to redissolve the gummy

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[4] THIS JOURNAL, **41**, 832 (1919).

precipitate locally formed by the alkali. To the filtered solution (about 350 cc.), powdered sodium nitrate was added to incipient turbidity and the mixture allowed to stand for several days, with occasional rubbing. The crystalline deposit weighed 28 g. after washing with 5% sodium nitrate solution. Recrystallized first from 25% alcohol, then from absolute alcohol, the nitrate forms faintly yellow rhombs which yield a white powder when ground. It is sparingly soluble in cold water, quite readily on boiling, the solution giving a blue-violet fluorescence with a little nitric or sulfuric acid and also giving the thalleoquinine test. When rapidly heated, the air-dry salt melts at 125–135° with effervescence and preliminary softening, but when anhydrous it softens above 90°, begins to melt at 95°, and is completely fluid at 115°. $[\alpha]_D^{27.5}$ of the anhydrous salt is +100.7 in 50% alcohol; $c = 1.063$.

Analyses. Calc. for $C_{20}H_{28}O_2N_2.HNO_3.H_2O$: H_2O, 4.40; C, 58.64; H, 7.64. Found: H_2O, 4.35; C, 58.73; H, 7.68.[5] Calc. for $C_{20}H_{28}O_2N_2.HNO_3$: N, 10.74. Found: 10.92.

d-Dihydroquinicinol.—The purified nitrate was converted into the base with alkali and shaken out with ether. After removing the solvent the warm residue was dissolved in a little hot benzene, with which it is miscible only if too much is not used. After cooling to room temperature and seeding with crystals obtained by allowing a test portion in benzene to evaporate spontaneously, the base crystallized rapidly as cream colored rhombs which were washed with a little cold benzene and air dried. It dissolves readily in alcohol, acetone or chloroform and turns gummy under dry ether or toluene, then dissolving with difficulty. It begins to sinter at 68° and is completely fluid at about 80°. $[\alpha]_D^{25}$ of the anhydrous base is + 87.1° in absolute alcohol; $c = 1.045$.

Analyses. Calc. for $C_{20}H_{28}O_2N_2.0.5H_2O$: H_2O, 2.66. Found: 2.34. Calc. for $C_{20}H_{28}O_2N_2$: C, 73.12; H, 8.60; N, 8.53. Found: C, 73.39; H, 8.63; N, 8.33.

d-Dihydroquinicinol Dihydrochloride.—An absolute alcoholic solution of the base was acidified to congo red with absolute alcoholic hydrochloric acid. The dihydrochloride gradually separated as rosets of minute needles which dissolve readily in water and rather sparingly in cold absolute alcohol, more easily on boiling. When rapidly heated to 210°, then slowly, the salt melts at 212–214° with gas evolution to form a yellow liquid. $[\alpha]_D^{25}$ is + 151.8° in water; $c = 1.096$.

Analysis. Calc. for $C_{20}H_{28}O_2N_2.2HCl$: Cl, 17.67. Found: Cl, 17.54.

l-Dihydroquinicinol Dihydrochloride.—In preliminary reduction experiments it was found that on taking up the crude base in absolute alcoholic hydrochloric acid an apparently homogeneous salt crystallized which, on recrystallization from absolute alcohol containing a little hydrochloric acid, finally attained a constant rotation of about +8°. Indeed it was from this salt that it was first found possible to obtain a portion as the *d*-nitrate, thus pointing to the initial isolation of this salt as the best method of separating the optical isomers. On adding more

[5] This analysis refers to a portion recrystallized again from 25% alcohol.

solid sodium nitrate to the filtrate from the first crop of the nitrate until turbid it is not always possible to obtain more of the nitrate free from an oily salt (probably the *l*-nitrate), but unless a considerable amount of the oil is precipitated with the remaining portions of the *d*-nitrate, some of the latter remains in solution, and on subsequent conversion of the filtrate into the dihydrochloride it is impossible to attain maximal levorotation.   Accordingly solid sodium nitrate was added to the filtrate from the first crop until considerable oily material separated, and after standing overnight the mixture was treated with bone black, filtered, and the dry base obtained from the filtrate in the usual manner. ˙ On dissolving in absolute alcohol containing an excess of dry hydrogen chloride and letting the mixture stand, the *l*-dihydrochloride separated on cooling as rosets of diamond-shaped, glistening platelets which then were repeatedly crystallized from absolute alcohol containing a little absolute alcoholic hydrochloric acid.   In this way a maximum $[\alpha]_D$ of $-117.7°$ was finally obtained in water; $c = 0.994$.   The salt is very hygroscopic in warm, moist air, and must therefore be filtered in a cold room.   It is very soluble in water and rather difficultly so in absolute alcohol, especially in the presence of dry hydrogen chloride.   Like the *d*-form it gives the thalleoquinine reaction and fluoresces on adding a suitable acid.   The free base was not obtained crystalline.   The salt slowly sinters together above 135° and effervesces at 170°.

Analyses.   Calc. for $C_{20}H_{28}O_2 N_2 . 2HCl$: C, 59.86; H, 7.49; N, 6.98.   Found: C, 59.22; H, 7.87; N, 7.67.

Essentially the same results were obtained when dihydroquinicine sulfate[6] was used as the starting material, except that in this case only one molecular equivalent of hydrogen was absorbed.

N-METHYL-DIHYDROQUINICINOL.

d-N-Methyl-dihydroquinicinol.—The crude quinicine base from 113.6 g. of quinidine methiodide[7] was dissolved in 300 cc. of 10% sulfuric acid and reduced with 35 cc. of 2% palladious chloride solution and hydrogen.   Approximately 2 molecular equivalents of hydrogen were absorbed.   The clear centrifuged liquid and washings were diluted, covered with ether, and made alkaline.   The residue from the dried ethereal extract crystallized on cooling, and was recrystallized from 50% alcohol, yielding 33 g. of crude *d*-N-methyl-dihydroquinicinol, or almost as much as if the intermediate quinicine salt[7] had actually been isolated. After 4 recrystallizations from 70% alcohol $[\alpha]_D^{17}$ was constant at $+93.9°$ in absolute alcohol, $= 1.012$, the yield being 8.3 g.   The base forms rhombic crystals which are anhydrous and melt at 165.5–166.0° with slight preliminary softening.   It is very readily soluble in chloroform, easily in dry methyl or ethyl alcohol, and sparingly in cold dry acetone or benzene but quite soluble on boiling.   Dissolved in conc. sulfuric acid it gives a faint yellow color which deepens and shows a green

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[6] See preceding paper, p. 223.

[7] Ref. 6, p. 221.

fluorescence when the solution is warmed on the water-bath. It gives the thalleo-quinine test and exhibits a violet-blue fluorescence when dissolved in dil. nitric or sulfuric acid.

Analyses. Calc. for $C_{21}H_{30}O_2N_2$: C, 73.63; H, 8.84; N, 8.19. Found: C, 73.47; H, 8.79; N, 8.34.

d-N-Methyl-dihydroquinicinol from *d*-Dihydroquinicinol Nitrate.—A dry acetone solution of *d*-dihydroquinicinol obtained from the nitrate was treated with a slight excess of methyl iodide. Heat was evolved, and when the solution was rubbed the N-methyl hydriodide separated as microscopic rhombs. The collected salt was dissolved in hot water, the solution cooled and treated with an excess of sodium carbonate. The base separated as an oil which soon crystallized on rubbing. After 3 recrystallizations from 70% alcohol $[\alpha]_D^{18}$ was +93.8° in absolute alcohol, $c = 1.002$, the melting point 165.5–166.0°, and the other properties also were identical with those of the base obtained from N-methyl-dihydroquinicine.

d-N-Methyl-dihydroquinicinol Hydrobromide.—A solution of the base in a slight excess of 10% aqueous hydrobromic acid was neutralized to litmus and the filtrate treated with solid sodium bromide until just turbid. The hydrobromide separated on chilling the solution and letting it stand. Recrystallized from water, it formed a hard crust of rhombs which were anhydrous, and turned pink and softened on heating, melting at 218–223° with darkening. It is rather sparingly soluble in cold water or absolute alcohol, but dissolves readily on heating. With a little sodium iodide or nitrate the aqueous solution soon deposits minute rosets of the iodide or rhombs of the nitrate. $[\alpha]_D^{14}$ is +80.2° in water; $c = 1.090$.

Analyses. Calc. for $C_{21}H_{30}O_2N_2 \cdot HBr$: N, 6.62; Br, 18.88. Found: N, 7.01; Br, 18.95.

d-N-Methyl-dihydroquinicinol Dihydrochloride.—This was obtained as in the case of *d*-dihydroquinicinol dihydrochloride. Recrystallized from absolute alcohol with the aid of dry ether it forms rosets of glistening platelets, which are extremely easily soluble in water, readily so in absolute alcohol, and practically insoluble in dry acetone. The salt softens markedly above 140°, gradually melting and evolving gas, and becoming completely fluid at about 190°. $[\alpha]_D^{14}$ is +145.7° in water; $c = 1.078$.

Analyses. Calc. for $C_{21}H_{30}O_2N_2 \cdot 2HCl$: N, 6.75; Cl, 17.08. Found: N, 6.91; Cl, 16.97.

d-N-Methyl-dihydroquinicinol Methiodide.—The oily salt separated from a solution of equimolecular parts of the constituents in chloroform, and soon crystallized. After washing with dry acetone it was recrystallized from water, by seeding the solution while still warm and letting it stand in a warm place until crystallization was almost complete, in order to avoid deposition of a gelatinous form. It forms a crust of faintly yellow rhombs which are very difficultly soluble in cold water or absolute alcohol, but more easily on boiling. When rapidly heated to 220°, then slowly, it begins to soften above this point and melts at 225–227°, to a brown turbid liquid which clears at 228°. $[\alpha]_D^{29.5}$ is +68.7° in water; $c = 1.025$.

Analyses. Calc. for $C_{22}H_{30}O_2N_2I$: I, 26.21. Found: 26.27.

l-N-Methyl-dihydroquinicinol.—The mother liquors from the first two recrystallizations of the crude N-methyl dihydroquinicinol were diluted to incipient turbidity and allowed to stand, when they deposited 8.4 g. of the crude *l*-compound with $[\alpha]_D$ −15° in absolute alcohol. After 2 recrystallizations from 60% alcohol 4.2 g. were obtained with $[\alpha]_D^{25}$ −24.9° in absolute alcohol, $c = 1.003$, the rotation being unchanged by further recrystallization. The *l*-base forms crusts of minute plates which melt with slight preliminary softening' at 136.5–137.5° to a liquid containing crystals, but becoming clear at 144°. It is more soluble in the usual organic solvents than the *d*-isomer, but otherwise greatly resembles this substance.

Analyses. Calc. for $C_{21}H_{28}O_2N_2$: C, 73.63; H, 8.84; N, 8.19. Found: C, 73.78; H, 8.50; N, 8.04.

The mother liquor from which the first crop of crude *l*-base was obtained still contained considerable amounts of base, which investigation showed to be a mixture.

l-N-Methyl-dihydroquinicinol Dihydrochloride.—This salt was obtained as in previous cases. Recrystallized from absolute alcohol, with the addition of a drop of absolute alcoholic hydrochloric acid after cooling, it separated as microscopic platelets. It is extremely easily soluble in water, readily so in dry methyl alcohol, sparingly soluble in cold absolute alcohol, and practially insoluble in dry acetone or chloroform. When rapidly heated to 230°, then slowly, it decomposes at 232–235°. The salt thus melts higher and is less soluble in absolute alcohol than the *d*-isomer. $[\alpha]_D^{24}$ is +1.45° in water; $c = 1.034$.

Analyses. Calc. for $C_{21}H_{30}O_2N_2.2HCl$: N, 6.75; Cl, 17.08. Found: N, 7.10; Cl, 17.06.

l-N-Methyl-dihydroquinicinol Methiodide.—The salt separated from a chloroform solution of the components. Recrystallized from water, it formed cream colored rosets of thick plates which are sparingly soluble in cold water, readily so in hot, and dissolve very difficultly in boiling absolute alcohol or dry chloroform or acetone. The iodide melts with preliminary browning and softening at about 253–254°, with decomposition. $[\alpha]_D^{25}$ is −50.0° in 50% alcohol; $c = 1.130$.

Analyses. Calc. for $C_{22}H_{33}O_2N_2I$: I, 26.21. Found: 26.42.

N-ETHYL-DIHYDROQUINICINOL.

d-N-Ethyl-dihydroquinicinol.—The crude quinicine from 93.5 g. of quinidine ethyl bromide[8] was reduced as in the case of the N-methyl compound. The crude base obtained from the ether extract was taken up in benzene and seeded with crystals obtained on concentration of a test portion of the crude base in ether. The *d*-base gradually crystallized, 12.8 g. being obtained after washing with a little benzene. Recrystallized twice from 70% alcohol it formed colorless, glistening rhombs which melted constantly at 140–141° and gave a constant $[\alpha]_D^{25}$ of

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[8] See preceding paper, p. 221.

+91.7° in absolute alcohol; $c = 1.030$. It is readily soluble in the cold in alcohol, methyl alcohol or chloroform, rather sparingly so in cold dry acetone, and difficultly soluble in cold benzene, but readily on boiling. In its reactions it greatly resembles the N-methyl derivative.

Analyses. Calc. for $C_{22}H_{32}O_2N_2$: C, 74.10; H, 9.05; N, 7.86. Found: C, 74.20; H, 8.88; N, 8.26.

d-N-Ethyl-dihydroquinicinol from *d*-Dihydroquinicinol Nitrate.—The base obtained from the nitrate was treated in acetone solution with 1.2 mols. of ethyl iodide. After several hours the hydriodide separated on rubbing as minute rosets, and was converted into the base. After 2 crystallizations from 70% alcohol this melted at 140–141° and had attained the maximum $[\alpha]_D^{25}$ of 92.0° in absolute alcohol; $c = 1.120$. In analysis and in its properties it also corresponded with the base as obtained directly.

d-N-Ethyl-dihydroquinicinol Hydrochloride.—Prepared as usual in absolute alcoholic solution the salt deposited after the addition of dry ether. Recrystallized from dry methyl ethyl ketone it formed minute rhombs which are very easily soluble in water. The solution has an intensely bitter taste. The salt dissolves readily in absolute alcohol, less easily in dry acetone, and sparingly in dry chloroform. When anhydrous, it softens at 130° and melts completely at 135° with slight decomposition. $[\alpha]_D^{20.5}$ in water is +85.4°; $c = 1.043$.

Analyses. Calc. for $C_{22}H_{32}O_2N_2.HCl.H_2O$: H_2O, 4.39. Found: 3.63. Calc. for $C_{22}H_{32}O_2N_2.HCl$: N, 7.13; Cl, 9.03. Found: N, 7.55; Cl, 8.97.

d - N - Ethyl - dihydroquinicinol Dihydrochloride. — The salt was obtained from absolute alcoholic hydrochloric acid with the aid of dry ether and was crystallized by a repetition of the process as minute platelets which are extremely soluble in water, readily in absolute alcohol, and sparingly so in dry acetone or chloroform; $[\alpha]_D^{22}$ is +142.6° in water; $c = 1.002$. It softens at about 150° and becomes semifluid, with the formation of gas bubbles at about 180° if slowly heated further, but if rapidly heated it melts and decomposes at about 250°.

Analysis. Calc. for $C_{22}H_{32}O_2N_2.2HCl$: N, 6 53; Cl, 16.52. Found: N, 6.79; Cl, 16.47.

A portion of the base recovered from the recrystallized salt had not increased in rotation.

d-N-Ethyl-dihydroquinicinol Methiodide.—A chloroform solution of the components was evaporated to dryness after one-half hour. The addition of dry acetone caused rapid crystallization. When recrystallized from water, the salt separated, oily at first if allowed to cool too quickly, but formed glistening platelets when the solution was seeded while still warm with crystals of the hydrate. When anhydrous it softens above 80°, melting completely at about 135°; $[\alpha]_D^{21}$ is +62.9° in 50% alcohol; $c = 0.962$. It also dissolves readily in absolute alcohol or dry chloroform.

Analyses. Calc. for $C_{23}H_{35}O_2N_2I.2H_2O$: H_2O, 6.74. Found: 6.31. Calc. for $C_{23}H_{35}O_2N_2I$: I, 25.48. Found: 25.90.

l-N-Ethyl-dihydroquinicinol Dihydrochloride.—In a preliminary experiment in which N-ethyl-dihydroquinicine hydrochloride[9] was used as starting material, the crude base was taken up in hot ligroin and the *d*-base allowed to separate from the warm solvent, cooling resulting in contamination with an oily fraction. The base recovered from the ligroin solution was converted as usual into the dihydrochloride. This was recrystallized from absolute alcohol with the aid of a little dry hydrogen chloride and dry ether, forming slightly brownish rosets which gave $[\alpha]_D^{25}$ $-16.7°$ in water; $c = 1.081$. When rapidly heated to 235°, then slowly, it decomposes at 237–238°. The amount obtained was insufficient for recrystallization to constant rotation. A portion, reconverted into the base, crystallized partially.

The fraction corresponding to the *l*-dihydrochloride in the larger experiment separated very quickly from absolute alcoholic hydrochloric acid on seeding, and was very difficult to filter. After recrystallization from absolute alcohol, to which dry hydrogen chloride was added after cooling, it again separated in very finely divided form, and as the base recovered from the salt showed little tendency to crystallize, further work on this considerable fraction was abandoned.

O-ETHYL-DIHYDROCUPREICINOL.

d-O-Ethyl-dihydrocupreicinol Hydrochloride.—The reduced solution of 20 g. of ethyl-dihydrocupreicine sulfate[9] was centrifuged from the palladium black, neutralized with sodium hydroxide, and treated with solid sodium chloride, until just turbid. The salt slowly crystallized. After several days in the ice-box it was filtered off, washed with 10% salt solution, and recrystallized from water, separating as cream colored prisms and needles. Further recrystallization from absolute alcohol yielded practically white plates containing alcohol of crystallization. The yield was 7.2 g. $[\alpha]_D^{26}$ of the dried salt was constant at $+81.1°$ in water, $c = 1.010$, and the melting point was 209–210° with preliminary softening. The salt is sparingly soluble in water or absolute alcohol at 0°, but dissolves more easily in methyl alcohol. An aqueous solution has a weakly bitter taste and a marked anesthetic action on the tip of the tongue. When treated with sodium bromide it soon deposits the bromide as sheaves and rosets of minute needles, and yields a nitrate of somewhat similar appearance with sodium nitrate.

Analyses. Calc. for $C_{21}H_{30}O_2N_2.HCl$: N, 7.40; Cl, 9.36. Found: N, 7.40; Cl, 9.26.

d-O-Ethyl-dihydrocupreicinol.—An aqueous solution of the salt was made alkaline with sodium carbonate. On letting stand, with occasional rubbing, the base gradually crystallized. When dissolved in alcohol and then well chilled and treated with water until the initial turbidity just redissolved, the base separates on seeding and standing in the ice-box as radiating masses of flat needles. If the recrystallization is attempted at room temperature the crystalline hydrate appar-

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[9] See preceding paper, p. 225.

ently does not form, and the base comes out oily. When washed with a little chilled 50% alcohol and air dried it comes to equilibrium with 2 molecules of water of crystallization. The hydrate dissolves readily in alcohol or acetone and melts at 59.5–61.5°. When anhydrous the base melts to a semifluid mass at 56–58°, with preliminary sintering and softening, and is completely fluid at 105–110°. $[\alpha]_D^{26.5}$ is +100.2° in absolute alcohol; $c = 1.018$.

Analyses. Calc. for $C_{21}H_{30}O_2N_2.2H_2O$: H_2O, 9.53. Found: 9.48. Calc. for $C_{21}H_{30}O_2N_2$: C, 73.63; H, 8.84; N, 8.19. Found: C, 73.97; H, 8.66; N, 8.67.

d-O-Ethyl-dihydrocupreicinol Dihydrochloride.—This was prepared from the monohydrochloride with alcoholic hydrochloric acid and ether. It forms rosets of platelets which are extremely soluble in water and rather sparingly so in absolute alcohol at room temperature but easily on heating. It gelatinizes under dry chloroform and dissolves with difficulty. When rapidly heated to 190°, then slowly, it melts with preliminary softening at 192–194° with gas evolution to a yellow liquid. $[\alpha]_D^{24}$ is + 149.2° in water; $c = 1.045$.

Analyses. Calc. for $C_{21}H_{30}O_2N_2.2HCl$: N, 6.75; Cl, 17.08. Found: N, 6.96; Cl, 16.96.

Neither the *l*-base nor any of its salts could be obtained crystalline.

d-N-Methyl-O-ethyl-dihydrocupreicinol.—*d*-O-Ethyl-dihydrocupreicinol hydrate was treated in dry acetone with an equivalent of methyl iodide. The N-methyl-hydriodide which separated as minute rosets and prismatic needles was converted into the base by addition of sodium carbonate to its aqueous solution. Recrystallized from 50% alcohol, it formed rosets of long, narrow, glistening platelets, which melted slowly at 136.5–137.0° with slight preliminary softening, giving $[\alpha]_D^{26.5}$ +88.2° in absolute alcohol, $c = 0.993$, and greatly resembled the corresponding dihydroquinicinol base in properties and reactions, except for a slightly greater solubility in organic solvents.

Analyses. Calc. for $C_{22}H_{32}O_2N_2$: C, 74.10; H, 9.05; N, 7.87. Found: C, 74.16; H, 8.93; N, 8.64.

d-N, O-Diethyl-dihydrocupreicinol.—Equimolecular amounts of the base and ethyl iodide were allowed to react in dry acetone, the hydriodide of the diethyl compound separating as sheaves of delicate needles. The base, obtained from the salt as in the preceding case, crystallized on adding a little ether and allowing this to evaporate. It was dissolved in hot 70% alcohol, cooled, and treated with water until just turbid, separating slowly when seeded and aided by the addition of occasional small portions of water as minute, glistening rhombs. The base melts constantly at 110–111° with slight preliminary softening and gives $[\alpha]_D^{19}$ +87.1° in absolute alcohol; $c = 0.804$. In its properties it resembles the methyl homolog, but is even more easily soluble.

Analyses. Calc. for $C_{23}H_{34}O_2N_2$: C, 74.54; H, 9.25; N, 7.57. Found: C, 74.87; H, 9.40; N, 7.68.

SUMMARY.

Cinchona alkaloids of the cinchonicine and quinicine type, reduced with palladium and hydrogen, yield mixtures of stereoisomers of a new type of alkaloids which we have called dihydrochonicinols and dihydroquinicinols. In general the *d*-forms proved easier to isolate. A number of the *d*- bases, one of the *l*-bases, and numerous salts of the *d*- and *l*- forms are described.

THE SYNTHESIS OF α-HYDROXYISOPENTACOSANIC ACID AND ITS BEARING ON THE STRUCTURE OF CERE-BRONIC ACID.

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Thudichum[1] was undoubtedly the first to isolate cerebronic acid. Unfortunately, he failed to recognize it as an oxy-acid and named it neurostearic acid. Thierfelder,[2] in 1904, recognized that cerebronic acid was an oxy-acid having the elementary composition $C_{25}H_{50}O_3$, but he gave no further details of its structure. In 1912, Levene and Jacobs[3] established the allocation of the hydroxyl group in the α-position to the carboxyl. The same conclusion was reached by Brigl in 1915. Regarding the further details of the structure of the acid two rival views were expressed. Levene and Jacobs[3] and Levene and West[4] had obtained on oxidation of cerebronic acid an acid of elementary composition, $C_{24}H_{48}O_2$. This acid melted at 81°C. The hydrocarbon obtained from this acid melted at 51–52°C. Since lignoceric acid is known to melt at 81°C., and the melting point of the hydrocarbon obtained from it was found to be 51–51.5°C., it was thought probable that the acid obtained on oxidation of cerbronic acid was lignoceric acid. Hence the structure of cerebronic acid was correlated to that of lignoceric acid.

Brigl[5] working under the direction of Thierfelder came to the conclusion that cerebronic acid had the structure of a normal acid. This theory was based on the following evidence. A normal α-hydroxy-pentacosanic acid was prepared and was found to melt at 102–104°C.

[1] Thudichum, J. L. W., Die Chemische Konstitution des Gehirns des Menschen und der Tiere, Tübingen, 1901, 194, 195.

[2] Thierfelder, H., *Z. physiol. Chem.*, 1904–05, xliii, 21.

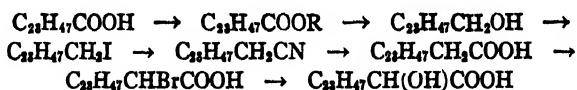[3] Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 381.

[4] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913, xiv, 257.

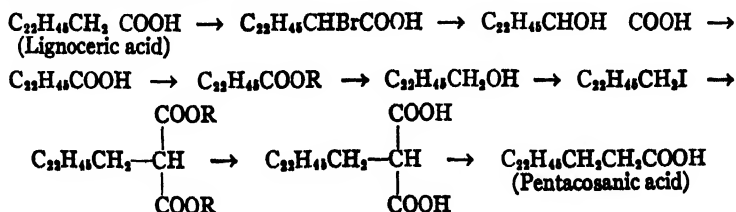[5] Brigl, P., *Z. physiol. Chem.*, 1915, xcv, 161.

An active cerebronic acid melting at 97–100°C. was inactivated. (Brigl does not give any experimental data regarding the method of racemization.) The melting point of the inactive acid remained unchanged. The mixed melting point of the synthetic acid and the inactivated natural acid was 98–100°C.

For conclusive proof of the view of Levene and his coworkers, there still lacked the proof through synthesis. α-Hydroxypentacosanic acid was prepared from lignoceric acid in order to compare it with the *dl*-cerebronic acid. The question naturally arose as to the method of racemization of cerebronic acid. The optical rotation observed on cerebronic acid is very low; namely, $[\alpha]_n^{20} = +4.16°$. Admixture of small proportions of this acid with the *dl*-form could scarcely be discovered. Hence it was thought of converting cerebronic acid into α-halogen acid which could then be reduced to a pentacosanic acid and this again converted into a *dl*-hydroxypentacosanic acid. It was, however, found impracticable to follow this plan and, therefore, cerebronic acid was oxidized to the acid $C_{24}H_{48}O_2$. This was then converted into hydroxypentacosanic acid.

The intermediate steps in the conversion of lignoceric acid and of the tetracosanic acid, from cerebronic acid, into α-hydroxypentacosanic acid, were identical, and as follows:

$$C_{22}H_{47}COOH \rightarrow C_{23}H_{47}COOR \rightarrow C_{22}H_{47}CH_2OH \rightarrow$$
$$C_{22}H_{47}CH_2I \rightarrow C_{22}H_{47}CH_2CN \rightarrow C_{22}H_{47}CH_2COOH \rightarrow$$
$$C_{22}H_{47}CHBrCOOH \rightarrow C_{23}H_{47}CH(OH)COOH$$

The melting points of the corresponding substances of each series, as well as the melting points of their mixtures, were identical. On the basis of these data, the evidence is conclusive that cerebronic acid is related to lignoceric acid. In order to render the proof more rigorous and to give greater confidence in the value of the melting points it was concluded to convert lignoceric acid into cerebronic acid by a second series of reactions as follows:

$$C_{22}H_{44}CH_2\ COOH \rightarrow C_{22}H_{44}CHBrCOOH \rightarrow C_{22}H_{44}CHOH\ COOH \rightarrow$$
(Lignoceric acid)

$$C_{23}H_{44}COOH \rightarrow C_{22}H_{44}COOR \rightarrow C_{22}H_{44}CH_2OH \rightarrow C_{22}H_{44}CH_2I \rightarrow$$

$$C_{22}H_{44}CH_2\!\!-\!\!\underset{\displaystyle COOR}{\overset{\displaystyle COOR}{CH}} \rightarrow C_{22}H_{44}CH_2\!\!-\!\!\underset{\displaystyle COOH}{\overset{\displaystyle COOH}{CH}} \rightarrow C_{22}H_{44}CH_2CH_2COOH$$
(Pentacosanic acid)

The melting point of this acid was identical with the corresponding acid obtained by the first process.

Another method commonly used for the identification of a fatty acid is its conversion into the corresponding hydrocarbon. Levene and Jacobs[3] and Levene and West[4] have converted cerebronic acid into a pentacosane by heating the acid with hydriodic acid. The product thus obtained melted at 53–54°C. However, it was realized that this method of preparing the hydrocarbon was not satisfactory. Therefore, lignoceric and the tetracosanic acids obtained from cerebronic acid, respectively, were converted into tetracosanes. Each melted at 51–51.5°C. It still seemed desirable to compare the two pentacosanes.

The pentacosanic acids prepared in the course of this work offered a convenient material for preparation of the pentacosanes corresponding to the pentacosanic acids from cerebronic and lignoceric acids by the following set of reactions:

$$C_{24}H_{49}COOR \rightarrow C_{24}H_{49}CH_2OH \rightarrow C_{24}H_{49}CH_2I \rightarrow C_{24}H_{49}CH_3$$
$$\text{(Pentacosane)}$$
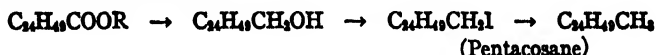
Every intermediate step in this set of reactions involves very mild treatment and every intermediate product can be readily purified. The melting points of the two hydrocarbons were identical; namely, 56–56.5°C. Again, on the ground of the melting points of the pentacosanes the relationship of cerebronic to lignoceric acid is confirmed. In connection with the pentacosane here described attention is called to the normal pentacosane prepared by Brigl.[5] The substance was prepared by the old Kraft method from the cetyloctylketone and melted at 55.5–56°C. If this melting point is correct for the normal pentacosane then the conversion of fatty acids with a very high number of carbon atoms to their respective hydrocarbons will have little value for the identification of fatty acids.

However, it is doubtful whether it is justifiable to rely on the absolute purity of the hydrocarbons prepared by Kraft's method from ketones. We intend to prepare normal pentacosane from normal pentacosanic acid by the same set of reactions as were used for the preparation of the isopentacosanes herein described.

The following table records all the melting points of the intermediate and final products prepared, respectively, from lignoceric acid and from tetracosanic acid obtained from cerebronic acid. (M.P. 99°C., $[\alpha]_D^{20} = +3.5°$ and M.P. 99.5°C., $[\alpha]_D^{20} = +3.8°$.)

Derivative of fatty acid.	M.P. of derivative of lignoceric acid from peanut oil.	M.P. of derivative from *d*-cerebronic acid.	M.P. of derivative of lignoceric acid from peanut oil.	M.P. of mixture.
	°C.	°C.	°C.	°C.
α-Bromolignoceric acid..................	68–69			
α-Hydroxylignoceric acid.................	91–92			
Isotricosanic acid.......................	73.5			
Ethyl isotricosanate....................	55.5			
Isotricosyl alcohol......................	69			
Isotricosy iodide........................	48			
Diethyl isotricosyl-malonate..............	52.5			
Isotricosyl-malonic acid..................	111			
Lignoceric acid.........................		80.5	81	80.5
Ethyl lignocerate.......................		55	55	55
Lignoceryl alcohol......................		72	72	72
Lignoceryl iodide.......................		48	48	48
Lignoceryl cyanide		56.5	56.5	56.5
Isopentacosanic acid.....................	78.5	78.5	78.5	78.5
α-Bromoisopentacosanic acid..............		70	70	70
α-Hydroxyisopentacosanic acid...........		92.5	92.5	92.5
Ethyl isopentacosanate..................		57	57	57
Isopentacosyl alcohol....................		75	75	75
Isopentacosyl iodide.....................		51.5	51.5	51.5
Isopentacosane.........................		56	56.5	56.5

From this table it is seen that the melting points of the *dl*-cerebronic acid prepared either from lignoceric acid or from tetracosanic acid obtained from cerebronic acid, melted at 92.5°C. The original cerebronic acid had approximately the same melting point as the cerebronic acids described by Thierfelder[2] and by Brigl.[5] The melting point of the inactivated cerebronic acid is given by Brigl 97–100°C. From this he attributes to cerebronic acid the structure of the normal acid for which the melting point is 102–104°C. It is evident on the basis of the results described here that Brigl has not succeeded in inactivating cerebronic acid by his process. In fact, the observation that the melting point lagged between 97–100°C. casts a suspicion on the purity of his substance. Also the normal hydroxypentacosanic acid, if it were pure, should be expected to melt more sharply than at 102–104°C. In a word, the data furnished by Brigl may be entirely disregarded in considering the structure of cerebronic acid. As evident from the present work this is *α-hydroxylignoceropentacosanic acid*.

There is one point that needs further investigation as a result of the present work. Thudichum, who was the first to isolate from phrenosin an acid having the elementary composition of hydroxypentacosanic acid, found its melting point at 84–85°C. Levene and Jacobs and Levene and West have also observed the same melting point of hydroxypentacosanic acid which was optically inactive. The low melting acid was regarded as the inactive cerebronic acid, perhaps in an impure state. Brigl[5] later expressed the view that the low melting acid was an isomer of cerebronic acid. Since the melting point of the *dl*-form of cerebronic acid is now established at 92.5°C., the question of the nature of the low melting acid needs further investigation.

EXPERIMENTAL.

In the following experiments, the melting points recorded were determined with a standardized Anschütz thermometer. The bath was continuously stirred and was heated by a burner regulated to raise the temperature 1° in 6 seconds.

Before analysis, each substance was dried to constant weight under a pressure of 0.5 mm. at the temperature of boiling chloroform.

The acid numbers were determined in mixtures of methyl alcohol (50 cc.) and benzene (25 cc.), using phenolphthalein as indicator. They are recorded as molecular weights.

Lignoceric Acid.—The acid from peanut oil which was used as the starting point for the following series of experiments was analytically pure and melted at 81°C. as given in the literature.

0.7286 gm. substance required 19.70 cc. 0.1 N NaOH.
0.1004 "   "   : 0.2880 gm. CO_2 and 0.1166 gm. H_2O.

$C_{24}H_{48}O_2$.   Calculated.   C 78.26, H 13.04, Mol. wt. 368.
           Found.   " 78.23, " 13.00, "   " 3.0.

α-Bromolignoceric Acid.—Dry, analytically pure lignoceric acid (50 gm.) was mixed with red phosphorus (6 gm.), melted on the water bath, and bromine (135 gm.) cautiously added. The heating was then còntinued for 6 to 7 hours. The hot, liquid acid bromide was decomposed in cold water and the α-bromolignoceric acid filtered off. In order to remove most of the water, the acid was dissolved in ether

and the solution filtered. The ether was removed by distillation and the residue twice crystallized from 50–60° petroleum ether. The acid was then analytically pure and melted at 68–69°C. Meyer, Brod, and Soyka,[6] after extensive purification, found a melting point of 68.5°C. The yield was 80 per cent of the theoretical.

0.1000 gm. substance: 0.2360 gm. CO_2 and 0.0958 gm. H_2O.

$C_{24}H_{47}O_2Br$.   Calculated.   C 64.39, H 10.59.
               Found.   " 64.36, " 10.72.

α-Hydroxylignoceric Acid.—α-Bromolignoceric acid (50 gm.) was hydrolyzed by heating on the water bath for 30 hours with 12 per cent sodium hydroxide solution (1,200 cc.). The mixture was vigorously stirred. When the operation was finished, the soap was filtered off and heated with dilute hydrochloric acid on the water bath until it liquefied. One crystallization from a liter of acetone usually sufficed to give an analytically pure product. The melting point was 91–92°C. as given by Meyer, Brod, and Soyka.[6] The yield was 95 per cent of the theoretical.

0.1035 gm. substance: 0.2854 gm. CO_2 and 0.1176 gm. H_2O.

$C_{24}H_{48}O_3$.   Calculated.   C 74.92, H 12.58.
             Found.   " 74.99, " 12.68.

Isotricosanic Acid.—This acid was prepared by the oxidation of α-hydroxylignoceric acid (50 gm.) in boiling acetone solution, with potassium permanganate, essentially as described by Levene and West[7] for the conversion of cerebronic into lignoceric acid. The finely divided permanganate (about 50 gm.) was dissolved in 2 liters of warm acetone. After all the permanganate had been introduced, the mixture was boiled until the pink color had disappeared. The solution was then cooled and filtered and the potassium salt of the isotricosanic acid separated from the manganese dioxide by three extractions with boiling 99.5 per cent alcohol (1 liter each). The salt which separated on cooling was decomposed by heating with dilute hydrochloric acid. After crystallizing the product from acetone and then from 99.5 per cent alcohol, it was analytically pure. The yield was 80 per cent of the

[6] Myer, H., Brod, L., and Soyka, W., *Monatsch. Chem.*, 1913, xxxiv, 1133.
[7] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913–14, xvi, 475.

theoretical. The acid melted at 73.5°C. after passing over the lead salt.

0.8048 gm. substance required 22.60 cc. 0.1 N NaOH.
0.0999 "   "   : 0.2854 gm. CO₂ and 0.1162 gm. H₂O.

$C_{23}H_{46}O_2$. Calculated. C 77.97, H 12.99, Mol. wt. 354.
        Found.   " 77.91, " 13.02, "   " 356.

Ethyl Isotricosanate.—Isotricosanic acid (30 gm.) was dissolved in absolute alcohol (200 cc.) together with sulfuric acid (9.5 gm.) and the solution boiled for 10 hours under a reflux condenser. The ester separated on cooling. One crystallization from 99.5 per cent alcohol gave an analytically pure product. The melting point of the ester was 55.5°C. The yield was 92 per cent of the theoretical.

0.1010 gm. substance: 0.2909 gm. CO₂ and 0.1176 gm. H₂O.

$C_{25}H_{50}O_2$. Calculated. C 78.53, H 13.09.
        Found. .   " 78.52, " 13.05.

Isotricosyl Alcohol.—The alcohol was prepared by the reduction of the corresponding ester in the manner described by Levene and Cretcher.[8] Because of the comparatively slight solubility of the ester, it was convenient to work with 10 gm. lots dissolved in a mixture of absolute alcohol (30 cc.) and dry toluene (15 cc.). Six times the calculated quantity of sodium was used. When the reduction was finished, the ethyl alcohol and toluene were removed in a current of steam, the soap and isotricosyl alcohol filtered off and extracted repeatedly with acetone. On cooling the acetone solution, the alcohol separated. It was purified by two further crystallizations from acetone and then melted at 69°C. The yield of analytically pure material was 76 per cent of the theoretical. Practically all of the remainder was recovered as isotricosanic acid.

0.1000 gm. substance: 0.2968 gm. CO₂ and 0.1286 gm. H₂O.

$C_{23}H_{48}O$. Calculated. C 81.09, H 14.21.
        Found.   " 80.94, " 14.39.

Isotricosyl Iodide.—The alcohol (20 gm.) was heated for 1 hour at 180°C. with 1.1 equivalents of iodine and an excess of red phosphorus.

[8] Levene, P. A., and Cretcher, L. H., Jr., *J. Biol. Chem.*, 1918, xxxiii, 505.

The product was twice crystallized from acetone and was then analytically pure. The iodide melted at 48°C. The yield was 95 per cent of the theoretical.

0.2024 gm. substance: 0.1046 gm. AgI (Carius).

$C_{23}H_{47}I$. Calculated. I 28.22.
        Found.   " 27.92.

Diethyl Isotricosyl-Malonate.—Isotricosyl iodide (20 gm.) was dissolved in absolute alcohol containing one equivalent of sodium ethylate and two equivalents of diethyl malonate. The solution was boiled on the water bath until neutral. The ester which separated on cooling was contaminated by a small quantity of isotricosyl iodide which was not entirely removed after a number of crystallizations. It melted at 52.5°C.

0.1000 gm. substance: 0.2724 gm. CO_2 and 0.1084 gm. H_2O.

$C_{30}H_{58}O_4$. Calculated. C 74.69, H 12.03.
        Found.   " 74.28, " 12.13.

Isotricosyl-Malonic Acid.—Crude isotricosyl-malonic ester was saponified by heating over night with a large excess of alcoholic sodium hydroxide on the water bath. The alcohol was then removed, the residue diluted with water, the crude soap filtered off and extracted with acetone. The soap was decomposed by heating with dilute hydrochloric acid on the water bath and the acid passed over the lead salt. It melted at 111°C. Nearly pure isotricosyl alcohol was recovered as a by-product from the acetone extract of the sodium salt.

0.0996 gm. substance: 0.2680 gm. CO_2 and 0.1026 gm. H_2O.

$C_{26}H_{50}O_4$. Calculated. C 73.24, H 11.74.
        Found.   " 73.38, " 11.53.

Isopentacosanic Acid.—The substituted malonic acid was heated at 180°C. as long as carbon dioxide was evolved. After one crystallization from acetone, the residue was analytically pure. The acid was passed over the lead salt and then melted at 78.5°C. The yield of the isopentacosanic acid from isotricosyl iodide was 93 per cent of the theoretical.

0.6904 gm. substance required 18.00 cc. 0.1 N NaOH.
0.1000 "   "   : 0.2880 gm. CO_2 and 0.1158 gm. H_2O.

$C_{25}H_{50}O_2$. Calculated. C 78.53, H 13.09, Mol. wt. 382.
Found.   " 78.54, " 12.96, "   " 384.

d-Cerebronic Acid.—Two samples of cerebrosides were hydrolyzed for the preparation of *d*-cerebronic acid. Both were of the less soluble material obtained on fractionation of the mixtures of crude cerebrosides isolated from brains and were practically pure phrenosin. Their rotations were determined in a solution of equal volumes of methyl alcohol and chloroform:

$$[\alpha]_D^{20} = \frac{+0.23° \times 100}{0.5 \times 4.10} = +11.2°$$

$$[\alpha]_D^{20} = \frac{+0.23° \times 100}{0.5 \times 4.77} = +9.7°$$

The cerebroside (500 gm.) was dissolved in 99.5 per cent alcohol (8,000 cc.) together with sulfuric acid (300 gm.) and the solution boiled for about 18 hours on the water bath. The ester crystallized at 0°C. It was recrystallized from acetone. The yield was 135 gm. It melted at 59.5°C. and had the following rotation in pyridine solution:

$$[\alpha]_D^{20} = \frac{+0.18° \times 100}{1.0 \times 7.20} = +2.5°$$

0.1003 gm. substance: 0.2793 gm. CO_2 and 0.1139 gm. H_2O.

$C_{27}H_{54}O_3$. Calculated. C 75.98, H 12.76.
Found.   " 75.91, " 12.71.

The other cerebroside, hydrolyzed under the same conditions and worked up in the same manner, gave rise to an ester which melted at 60°C. and showed the following rotation:

$$[\alpha]_D^{20} = \frac{+0.25° \times 100}{1.0 \times 5.56} = +4.5°$$

0.1004 gm. substance: 0.2790 gm. CO_2 and 0.1165 gm. H_2O.

$C_{27}H_{54}O_3$. Calculated. C 75.98, H 12.76.
Found.   " 75.78, " 12.99.

The cerebronic ester was saponified by boiling its alcoholic solution with a large excess of sodium hydroxide on the water bath over night.

The alcohol was removed and the soap decomposed in the usual manner. The cerebronic acid was crystallized from acetone and passed over the lead salt. It was then analytically pure and melted at 99°C. Fractionation by means of the lithium salt did not separate any lignoceric acid. The rotation in pyridine was

$$[\alpha]_D^{20} = \frac{+0.29° \times 100}{1.0 \times 8.38} = +3.5°$$

0.7303 gm. substance required 18.27 cc. 0.1 N NaOH.
0.0997 "   "   : 0.2754 gm. CO_2 and 0.1106 gm. H_2O.

$C_{25}H_{50}O_3$. Calculated. C 75.38, H 12.56, Mol. wt. 398.
Found.   " 75.33, " 12.42, "   "   400.

The second cerebronic ester, subjected to the same procedure, gave rise to an acid which melted at 99.5°C. It was analytically pure and had the following rotation:

$$[\alpha]_D^{20} = \frac{+0.33° \times 100}{1.0 \times 8.79} = +3.8°$$

0.7346 gm. substance required 18.45 cc. 0.1 N NaOH.
0.1004 "   "   : 0.2787 gm. CO_2 and 0.1124 gm. H_2O.

$C_{25}H_{50}O_3$. Calculated. C 75.38, H 12.56, Mol. wt. 398.
Found.   " 75.70, " 12.53, "   "   398.

Lignoceric Acid.—The cerebronic acid (25 gm.) was oxidized in acetone solution. The procedure followed exactly that described for the oxidation of α-hydroxylignoceric acid to isotricosanic acid. The yield was 81 per cent of the theoretical. It was passed over the lithium and lead salts and then melted at 80.5°C.

0.7227 gm. substance required 19.42 cc. 0.1 N NaOH.
0.0999 "   "   : 0.2863 gm. CO_2 and 0.1166 gm. H_2O.

$C_{24}H_{48}O_2$. Calculated. C 78.26, H 13.04, Mol. wt. 368.
Found.   " 78.15, " 13.06, "   "   372.

The following descriptions represent two series of experiments. In one the starting point was the lignoceric acid from cerebronic acid, described above. In the second, the lignoceric acid from peanut oil, described at the beginning of the experimental part, served as the starting point. In each experiment one melting point is given. The mixed melting points are tabulated at the end of the introduction.

Analytical data are given for both series; those of the members of the cerebroside series under (A) and of the others under (B).

Ethyl Lignocerate.—A solution of lignoceric acid (30 gm.) and sulfuric acid (10 gm.) in 99.5 per cent alcohol (500 cc.) was boiled over night on the water bath. The ester separated at 0°C. Crystallization from 99.5 per cent alcohol gave an analytically pure product. The yield was 96 per cent of the theoretical. The ester melted at 55°C. as given in the literature.

(A) 0.1001 gm. substance: 0.2888 gm. CO_2 and 0.1166 gm. H_2O.
(B) 0.1006 "   " : 0.2894 " " " 0.1182 "   "

$C_{26}H_{52}O_2$.   Calculated.   C 78.79, H 13.13.
(A)   Found.   " 78.68, " 13.02.
(B)   "   " 78.45, " 13.15.

Lignoceryl Alcohol.—Ethyl lignocerate (10 gm.) was reduced with sodium and absolute alcohol. The process has already been described. Two crystallizations from acetone sufficed to give an analytically pure substance. 83 per cent of the ester was recovered as the alcohol and practically all of the remainder as lignoceric acid. The alcohol melted at 72°C. as given in the literature.

(A) 0.1006 gm. substance: 0.2990 gm. CO_2 and 0.1268 gm. H_2O.
(B) 0.1004 "   " : 0.2987 " " " 0.1262 "   "

$C_{24}H_{50}O$.   Calculated.   C 81.36, H 14.12.
(A)   Found.   " 81.05, " 14.11.
(B)   "   " 81.13, " 14.06.

Lignoceryl Iodide.—The alcohol (18 gm.) was heated with red phosphorus and iodine at 180°C. for 1 hour. The product was twice crystallized from acetone. It then melted at 48°C., as given in the literature, and was analytically pure. The yield was 96 per cent of the theoretical.

(A) 0.2010 gm. substance: 0.1024 gm. AgI (Carius).
(B) 0.2014 "   " : 0.1032 " " "

$C_{24}H_{49}I$.   Calculated.   I 27.37.
(A)   Found.   " 27.53.
(B)   "   " 27.69.

Lignoceryl Cyanide.—Lignoceryl iodide (10 gm.) was dissolved in 99.5 per cent alcohol (300 cc.) and finely pulverized potassium cyanide (11.5 gm.) added. The solution was boiled on the water bath for about 17 hours. The nitrile which separated at 0°C., was crystallized from acetone. It melted at 56.5°C.

> (A) 0.2000 gm. substance required 5.20 cc. 0.1 N HCl.
> (B) 0.2000 "   "   "   5.00 " 0.1 N "

> $C_{25}H_{49}N$. Calculated.   N 3.86.
> (A)   Found.   " 3.64.
> (B)   "   " 3.50.

For the preparation of isopentacosanic acid the nitrile was not isolated.

Isopentacosanic Acid.—All of the ether-soluble material from the preparation of the cyanide was dissolved in 99.5 per cent alcohol with a large excess of sodium hydroxide. The solution was boiled on the water bath for 24 hours. The alcohol was then evaporated, the residue extracted with boiling acetone, and the insoluble soap decomposed with dilute hydrochloric acid on the water bath. The product was analytically pure after one crystallization from acetone. The yield of isopentacosanic acid from the lignoceryl iodide was 93 per cent of the theoretical. It melted at 78.5°C.

> (A) 0.7621 gm. substance required 19.80 cc. 0.1 N NaOH.
> (B) 0.7839 "   "   "   20.30 " 0.1 N "
> (A) 0.1002 "   "   : 0.2882 gm. CO_2 and 0.1168 gm. H_2O.
> (B) 0.1004 "   "   : 0.2896 "   "   "   0.1159 "   "

> $C_{25}H_{50}O_2$. Calculated.   C 78.54, H 13.09, Mol. wt. 382.
> (A)   Found.   " 78.43, " 13.04, "   " 385.
> (B)   "   " 78.66, " 12.91, "   " 386.

α-Bromoisopentacosanic Acid.—Isopentacosanic acid (7.0 gm.) was melted on the water bath with red phosphorus (0.4 gm.). Bromine (11.0 gm.) was then slowly introduced and the mixture heated for 4 hours. An excess of bromine was present at the end. The acid bromide was decomposed, the acid dried and crystallized from 40–50° gasoline. The yield of pure acid was 95 per cent of the theoretical. It melted at 70°C.

(A) 0.2024 gm. substance: 0.0808 gm. AgBr (Carius).
(B) 0.1980 "   "   : 0.0800 "   "   "

$C_{25}H_{47}O_2Br$.   Calculated.   Br 17.32.
(A)   Found.   " 16.99.
(B)   "   " 17.20.

dl-Cerebronic Acid.—α-Bromoisopentacosanic acid (50 gm.) was suspended in 12 per cent aqueous sodium hydroxide (200 cc.) and heated on the water bath for 40 hours with violent stirring. The soap was then decomposed with dilute hydrochloric acid on the water bath and crystallized from acetone. After the acid had been twice crystallized from chloroform and passed over the lead salt, it melted at 92.5°C. The yield of analytically pure material was 93 per cent. The yield of *dl*-acid from the naturally occurring *d*-cerebronic acid was approximately 60 per cent.

(A) 0.8090 gm. substance required 20.35 cc. 0.1 N NaOH.
(B) 0.9150 "   "   "   22.81 " 0.1 N "
(A) 0.1004 "   "   : 0.2782 gm. CO_2 and 0.1107 gm. H_2O.
(B) 0.1004 "   "   : 0.2784 "   "   " 0.1154 "   "

$C_{25}H_{50}O_3$.   Calculated.   C 75.38, H 12.56, Mol. wt. 398.
(A)   Found.   " 75.56, " 12.34, "   " 398.
(B)   "   " 75.61, " 12.86, "   " 401.5.

Ethyl Isopentacosanate.—Isopentacosanic acid (15 gm.) was dissolved in 99.5 per cent alcohol (150 cc.) together with sulfuric acid (5 gm.) and the solution boiled on the water bath over night. The ester which separated at 0°C. was twice crystallized from 99.5 per cent alcohol. The yield of analytically pure ester was 98 per cent. It melted at 57°C.

(A) 0.1003 gm. substance: 0.2899 gm. CO_2 and 0.1158 gm. H_2O.
(B) 0.1004 "   "   : 0.2916 "   "   " 0.1212 "   "

$C_{27}H_{54}H_2$.   Calculated.   C 78.95, H 13.26.
(A)   Found.   " 78.82, " 12.92.
(B)   "   " 79.20, " 13.50.

Isopentacosyl Alcohol.—The ester was reduced by means of sodium and alcohol in the manner already described for isotricosyl alcohol. Two crystallizations from acetone sufficed to give an analytically pure product. The yield was 92 per cent. The alcohol melted at 75°C.

(A) 0.1001 gm. substance: 0.2992 gm. CO_2 and 0.1269 gm. H_2O.
(B) 0.0998   "   "   : 0.2987 "   "   " 0.1282 "   "

$C_{25}H_{52}O$.	Calculated.	C 81.45, H 14.22.
(A)	Found.	" 81.51, " 14.19.
(B)	"	" 81.62, " 14.37.

Isopentacosyl Iodide.—The alcohol was heated at 180°C. for 1 hour with iodine and red phosphorus. After two crystallizations from acetone, it was analytically pure and melted at 51.5°C. The yield was 98 per cent.

(A) 0.2026 gm. substance: 0.1002 gm. AgI (Carius).
(B) 0.2088   "   "   : 0.1018 "   "   "

$C_{25}H_{51}I$.	Calculated.	I 26.53.
(A)	Found.	" 26.73.
(B)	"	" 26.34.

Isopentacosane.—Isopentacosyl iodide (8 gm.) was dissolved in hot glacial acetic acid (300 cc.) and the solution saturated with dry hydrogen chloride. Zinc dust was then added in small portions at intervals to maintain a vigorous evolution of hydrogen. The reduction was continued for 48 hours. The product was poured into water and the hydrocarbon filtered off. It was analytically pure after two crystallizations from 99.5 per cent alcohol. It then melted at 54°C. Crystallization from ether finally raised the melting point to 56°C. The yield of hydrocarbon from *d*-cerebronic acid was about 60 per cent of the theoretical.

(A) 0.1010 gm. substance: 0.3153 gm. CO_2 and 0.1329 gm. H_2O.
(B) 0.1002   "   "   : 0.3126 "   "   " 0.1324 "   "

$C_{25}H_{52}$.	Calculated.	C 85.13, H 14.87.
(A)	Found.	" 85.13, " 14.72.
(B)	"	" 85.08, " 14.79.

ON A POSSIBLE ASYMMETRY OF ALIPHATIC DIAZO COMPOUNDS. II.

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It has long been known that the esters of optically active amino-acids on treatment with nitrous acid give rise to esters of the corresponding hydroxy-acids. Likewise esters or inner esters (lactones) of amino sugar acids give rise not to a mixture of the esters of two epimeric hydroxy-acids, but to one single substance. It has also been known for some time that on mild treatment of the esters of amino-acids with nitrous acid, diazo derivatives and not hydroxy-acid esters are formed.

Levene and La Forge[1] in 1915 made the observation that the esters of benzilidine ethyl chitosaminate on mild treatment with nitrous acid gave rise to a diazo derivative. This observation was an impetus to a scrutiny into a possible connection between the formation of the diazo derivative and the hydroxy-acid. It seemed possible to regard the diazo derivative as an intermediary phase in the transformation of the esters of amino-acids into the esters of hydroxy-acids. If this were proven it would necessitate the assumption of the existence of enantiomorphous aliphatic diazo esters.

This part of the problem required experimental verification. Work was in progress in this laboratory for several years on substances of the two mentioned groups. In 1920 Marvel and Noyes[2] criticized the deductions which Levene made from his observations on the behavior of hexosaminic acids. According to Marvel and Noyes the formation of one hydroxy-acid (and not a pair of epimeric acids) may be readily explained by the presence of four asymmetric carbon atoms in the molecule of these substances and on the assumption that the

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 345.
[2] Marvel, C. S., and Noyes, W. A., *J. Am. Chem. Soc.*, 1920, xlii, 2259.

configuration of carbon atoms 3, 4, and 5 control the configuration of carbon atom 2. That the objections of Marvel and Noyes are not valid is readily seen from the following graphic expressions:

I

COOR
|
H C NH$_2$
|
OH C H
|
H C OH
|
H C OH
|
C H$_2$OH

(Glucose aminic ester)

→

II

COOR
|
C (N‖N)
|
OH C H
|
H C OH
|
H C OH
|
C H$_2$OH

(Diazo derivative)

←

III

COOR
|
NH$_2$ C H
|
OH C H
|
H C OH
|
H C OH
|
C H$_2$OH

(Mannose aminic ester)
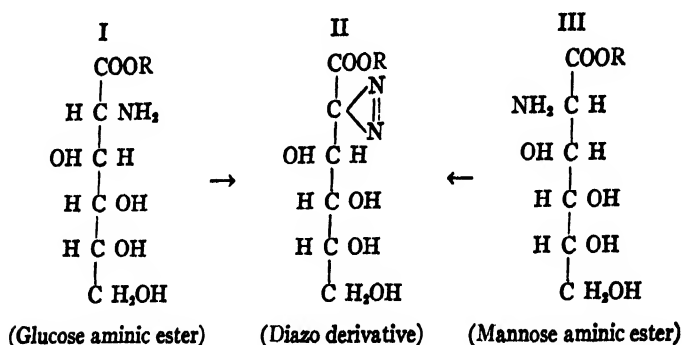
The configuration of carbon atoms 3, 4, and 5 are identical in the two diazo derivatives and if the configuration of carbon atom 2 in the acid derived from (I) is different from that derived from (III) then the resulting configurations of carbon atom 2 were brought about by other factors than the configuration of carbon atoms 3, 4, and 5. Hence any conclusions reached on the basis of experiments on one group of substances may be applied also to the other group.

In 1920–21 a brief report[3] was published on the diazo ester obtained from d-aspartic ester. The evidence contained in it is suggestive of the possibility of the existence of optically active diazosuccinic ester. The publication of the details was delayed, as it was our intention to postpone it until the preparation of a crystalline aliphatic diazo derivative was accomplished. External consideration, however, prompts us to publish the details of the work on the diazodiethyl succinates at this date.

The results of the analysis in the previous communication and the optical rotation of the diazo esters have already been reported. The substance was prepared by the method of Curtius and purified by distillation at a pressure of not more than 0.1 mm. By further fractionation it was not possible to separate the material into fractions differing in their optical activity. The highest optical activity observed was $[\alpha]_D^{20} = 1.34°$.

[3] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1920–21, xlv, 593.

It was not excluded that the substance was a mixture of the d and dl forms. There was, in fact, evidence for the assumption that the material is easily inactivated. Thus in some experiments the diazo esters could not be distilled unless previously dried by barium oxide. On such treatment the product generally darkened and on distillation yielded a diazo ester of much lower optical activity than the samples which did not require the treatment with barium oxide.

In order to find more rigorous proof for the existence of an optically active diazodiethyl succinate, the material was converted into diethyl malate. Since the optical rotation of the unracemized diazo derivative is not known and since the specific rotation of diethyl malate is much higher than the one observed for the diazo derivative, it was reasoned that an increase in optical activity after hydrolysis should be interpreted in the sense of the conversion of diazo derivative into an optically active hydroxy ester. The result of the experiment seemed to point in the desired direction, since the rotation of the material obtained from hydrolysis of the diazo ester was higher than the rotation of the original material. However, the increase was not sufficiently great to render the evidence conclusive.

In view of this, the diazo ester was converted into the halogen acid esters by the action of hydrobromic or hydrochloric acid. If under such conditions active halogen succinic esters were obtained this finding could be interpreted only in the sense of the existence of an optically active diazo ester. Optically active esters of the chloro- and bromosuccinic esters were prepared. The analysis of the bromo derivative was quite satisfactory; the theory requiring Br = 31.60 per cent and the value found being Br = 31.12 per cent. The optical rotation of the material was $[\alpha]_D^{20} = + 0.40° (\pm 0.00)$.

The choroethyl succinate analyzed as follows:

Calculated. C 46.11, H 6.24, Cl 17.01.
Found.   "   46.24, "   7.04, "   15.35.

The optical rotation was $[\alpha]_D^{20} = + 0.86° (\pm 0.00)$. The low value of chlorine can be explained by the presence in the material of either malic or fumaric esters. The material was therefore hydrogenated in the presence of colloidal palladium and it absorbed an amount of hydrogen which would be required by the presence of 10.23 per cent

of fumaric acid. Thus the discrepancy between the theoretical and found values of chlorine may be explained on the basis of the presence of fumaric acid, in which case the chloromalic acid is optically active.

Thus, all evidence points towards the existence of optically active diazodiethyl succinate. However, further evidence is required for the final solution of the problem. Work in this direction is in progress.

EXPERIMENTAL.

Nitrogen Estimation.—It was found convenient to use Van Slyke's amino apparatus. For this purpose a leveling bulb was connected to the lower outlet of the mixing chamber by a rubber tube, and filled with mercury. Before beginning the analysis the mixing chamber is filled with mercury. By lowering the leveling bulb a vacuum is created. 25 per cent sulfuric acid is then introduced into the mixing chamber and then the diazo derivative dissolved in a mixture of equal parts of isopropyl alcohol and water. On gentle shaking the operation is completed in about 2 minutes. The further procedure is the same as in Van Slyke's method for amino nitrogen estimation.

By this method diazoethyl acetate and diazoethyl succinamide were analyzed with the following results:

2 cc. solution of 0.2990 gm. diazoethyl acetate in 10 cc. isopropyl alcohol and water 1 : 1 gave in Van Slyke apparatus 1.11 cc. N_2, $P = 770.8$ mm., $t = 23°C$.

$$C_4H_6O_2N_2. \quad \text{Calculated.} \quad N \quad 20.74$$
$$\text{Found.} \quad `` \quad 21.10$$

2 cc. solution of 0.7200 gm. diazoethyl succinamide in 10 cc. water gave in Van Slyke apparatus 2.09 cc. N_2, $P = 762.2$ mm., $t = 24°C$.

$$C_6H_9O_2N_3. \quad \text{Calculated.} \quad N \quad 16.43$$
$$\text{Found.} \quad `` \quad 16.24$$

To test further this method of analysis, on several occasions the analysis was controlled by an estimation by the Dumas method. For the latter purpose the substance was weighed in a very fine capillary and the further analysis was completed in the usual way. The results of the comparative analyses were as follows.

2 cc. solution of 0.6760 gm. diazodiethyl succinate in 10 cc. 1 : 1 isopropyl alcohol gave in Van Slyke apparatus 1.60 cc. N_2, $P = 764$ mm., $t = 24°C$.

0.0978 gm. substance gave, Dumas, 12 cc. N_2, $P = 764$ mm., $t = 26°C$.

$C_8H_{12}O_4N_2$ Calculated.   N   14.00.
             Found (Van Slyke).   "   13.93.
                  "   (Dumas).   "   14.07.

Preparation of Diazodiethyl Succinate.—Much difficulty was experienced in the purification of this ester, as the least trace of water or some other impurity seemed to interfere with distillation. Methods of treatment had to be changed at times to make distillation possible. Some of these will be described below.

60 gm. of diethyl aspartate hydrochloride ($[\alpha]_D^{20} = +8.15°$) were dissolved in 90 cc. of water and cooled to 0°. To this was added a solution of 45 gm. of sodium nitrite in 90 cc. of water and also about 200 cc. of ether. The mixture was thoroughly cooled in a freezing mixture (salt and ice), whereupon cold 25 per cent sulfuric acid was gradually added. The ether layer was drained off, more ether was added, which was followed by the addition of more acid. This procedure was repeated until the ether extract was practically colorless. The ether extract was then washed with a cold saturated solution of sodium carbonate until the aqueous layer, which in the first washing turned red, remained practically colorless. The ether solution was then washed with distilled water and dried by allowing it to stand over anhydrous sodium sulfate for several hours.

The ether was subsequently removed by distilling under reduced pressure. An attempt to distill the residue was unsuccessful since the high vacuum could not be maintained. The ester was therefore redissolved in ether and treated with anhydrous sodium sulfate. After about 2 hours the ether was removed and the residue distilled. Two fractions were obtained, boiling at 75–80°C., at a pressure of about 0.12 mm.

2 cc. solution of 0.0984 gm. Fraction I in 10 cc. isopropyl alcohol and water 1 : 1 gave 2.35 cc. N_2, $P = 766.4$ mm., $t = 21°C$.

2 cc. solution of 0.0714 gm. Fraction II in 10 cc. isopropyl alcohol and water 1 : 1 gave 1.78 cc. N_2, $P = 766.4$ mm., $t = 21°C$.

		N_2 per cent	Impurity. per cent
$C_8H_{12}O_4N_2$.	Calculated.	14.00	
	Found, Fraction I.	13.64	2.5
	" " II.	14.20	0.0

Fraction I. $[\alpha]_D^{20} = \dfrac{+0.33° \times 100}{1 \times 32.9} = +1.00°$

Fraction II. $[\alpha]_D^{20} = \dfrac{+0.47° \times 100}{1 \times 35.1} = +1.34°$

In subsequent preparations of the pure diazo derivative it was found impossible to distill the material without further treatment. In some cases it was found necessary to treat it with anhydrous potassium carbonate.   In other instances this did not suffice but a further treatment with calcium hydroxide was necessary.   Barium oxide was also used with success.   In the experiments in which calcium hydroxide was used the optical activity of the pure diazodiethyl succinate was found to be

$$[\alpha]_D^{20} = \frac{+0.22° \times 100}{0.5 \times 100} = +0.44°$$

Found.   N 14.26 and 14.20 per cent.

In another experiment in which calcium hydroxide was used the optical activity was found to be

$$[\alpha]_D^{20} = \frac{+0.25° \times 100}{0.5 \times 100} = +0.50°$$

Found.   N 14.04 per cent.

Conversion of Diazodiethyl Succinate into Hydroxy Succinate.— About 5 gm. of pure diazodiethyl aspartate were dissolved in 50 cc. of ether and cooled by means of a freezing mixture.   25 per cent sulfuric acid was then gradually added, with shaking, until the yellow color disappeared.   The ether layer was separated, and the mother liquor extracted with ether several times.   The ether extracts were combined, dried with sodium sulfate, and the ether was removed under diminished pressure.   The residue weighed 3.01 gm.   This was diluted to 10 cc. with ether and the optical activity determined.

$$[\alpha]_D^{20} = \frac{+0.16° \times 100}{1 \times 30.1} = +0.53°$$

The reading of the original diazo derivative without solvent was

$$[\alpha]_D^{20} = \frac{+0.22° \times 100}{0.5 \times 100} = +0.44°$$

Diazo nitrogen in the original diazo derivative, as already mentioned, was N = 14.26 and 14.20 per cent.

In another experiment a larger quantity of diazo ester was prepared and hydrolyzed, without previous distillation. The original material had an optical rotation $[\alpha]_D^{18} = +3.84°$. The ethyl malate was prepared in the usual way and the product fractionated as follows:

5.82 gm. Fraction I, boiling at 114–117°C., made up to 10 cc. with ether gave

$$[\alpha]_D^{18} = \frac{+1.11° \times 100}{1 \times 58.2} = +1.90°$$

5.36 gm. Fraction II, boiling at 127–136°C., made up to 10 cc. with ether gave

$$[\alpha]_D^{18} = \frac{+1.60° \times 100}{1 \times 53.6} = +2.98$$

4.07 gm. Fraction III, boiling at 136–160°C., made up to 10 cc. with ether gave

$$[\alpha]_D^{18} = \frac{+2.88° \times 100}{1 \times 40.7} = +7.07°$$

This material contained, in addition to the diethyl malate, partly hydrolyzed material and some fumaric ester, which had been isolated on many occasions. The partial saponification was made evident on another material which happened to have a lower optical rotation. The fractions obtained on first distillation showed values for carbon which were too low for the esters, which could be explained on the basis of partial saponification. After several fractionations a product was obtained which analyzed correctly for diethyl malate, as follows:

0.1006 gm. substance: 0.1854 gm. CO_2 and 0.0670 gm. H_2O.
$C_8H_4O_5$.   Calculated.   C 50.52, H 7.37.
          Found.   " 50.26, " 7.45.

Conversion of Diazodiethyl Succinate into Monobromodiethyl Succinate.—180 gm. of diethyl aspartate hydrochloride were diazotized as in the preparation of the diazo derivative. The diazo ester was washed with a solution of sodium carbonate and dried over sodium sulfate. Without further purification the ethereal solution of the diazo ester was cooled and then slowly added to a cold saturated solution of hydrobromic acid gas in ether. After the evolution of gas had ceased the reaction mixture was of a dark brown color which again turned to straw yellow on washing with an aqueous sodium carbonate. The ethereal solution was then washed with distilled water, dried

over sodium sulfate, and the ether removed under diminished pressure. The residue was then subjected to fractional distillation:

Fraction No.	Boiling point.	Pressure.	$[\alpha]_D^{20}$	Br content.
	°C.	mm.		per cent
I	75–80	0.134	$\dfrac{+0.22° \times 100}{0.5 \times 100} = +0.44°$	Not determined.
II	76–79	0.067	$\dfrac{+0.25° \times 100}{0.5 \times 100} = +0.50°$	28.40
III	77–79	0.040	$\dfrac{+0.24° \times 100}{0.5 \times 100} = +0.48°$	30.10
IV	75–77	0.067	$\dfrac{+0.20° \times 100}{0.5 \times 100} = +0.40°$	30.56
V	74–76	0.094	$\dfrac{+0.16° \times 100}{0.5 \times 100} = +0.32°$	30.51

Fractions IV and V were combined and redistilled:

Fraction No.	Boiling point.	Pressure.	$[\alpha]_D^{20}$	Br content.
	°C.	mm.		per cent
I	66–70	0.037	$\dfrac{+0.20° \times 100}{0.5 \times 100} = +0.40°$	30.36
II	68–70	0.023	$\dfrac{+0.20° \times 100}{0.5 \times 100} = +0.40°$	30.99
III	69–70	0.023	$\dfrac{+0.20° \times 100}{0.5 \times 100} = +0.40°$	30.74
IV	69–70	0.023	$\dfrac{+0 20° \times 100}{0.5 \times 100} = +0.40°$	30.51

Fractions II and III were combined and redistilled:

Fraction No.	Boiling point.	Pressure.	$[\alpha]_D^{20}$	Br content.
	°C.	mm.		per cent
I	80–81	0.040	$\dfrac{+0.20° \times 100}{0.5 \quad \times 100} = +0.40°$	30.82
II	81–82	0.046	$\dfrac{+0.20° \times 100}{0.5 \quad \times 100} = +0.40°$	31.12
III	81–82	0.047	$\dfrac{+0.20° \times 100}{0.5 \quad \times 100} = +0.40°$	30.68

0.2058 gm. substance: 0.1500 gm. AgBr.

$C_8H_{13}O_4Br.$ Calculated.   Br   31.60.
                Found.   "   31.12.

Conversion of Diazodiethyl Succinate into Monochlorodiethyl Succinate.—Diazoethyl succinate was dissolved in 5 volumes of dry ether and the solution treated with dry HCl gas with cooling. After the evolution of nitrogen had ceased the ether was distilled off under reduced pressure and the optical activity of the residue determined. It was found to be:

$$[\alpha]_D^{20} = \frac{+0.36° \times 100}{0.5 \times 100} = +0.72°$$

The original diazo derivative showed:

$$[\alpha]_D^{20} = \frac{+0.25° \times 100}{0.5 \times 100} = +0.50°$$

The chlorester was fractionated as follows:

Fraction No.	Boiling point.	Pressure.	$[\alpha]_D^{20}$
	°C.	mm.	
I	61–68	0.050	$\dfrac{+0.18° \times 100}{0.5 \quad \times 100} = +0.36°$
II	67–68	0.048	$\dfrac{+0.25° \times 100}{0.5 \quad \times 100} = +0.50°$
III	68–70	0.057	$\dfrac{+0.43° \times 100}{0.5 \quad \times 100} = +0.86°$

Fraction III was analyzed:

0.1004 gm. substance: 0.0580 gm. H₂O and 0.1900 gm. CO₂.

         $C_8H_{13}O_4Cl$.   *Calculated.*   C 46.11,   H 6.24,   Cl 17.01.

                 Found.   " 46.18,   " 6.47,   " 15.35.

0.5602 gm. Fraction III was dissolved in ether and reduced with hydrogen in the presence of colloidal palladium. The reduction was continued until no more hydrogen was being absorbed. 8.00 cc. hydrogen were absorbed. *t* = 22°C., *P* = 752 mm.

The amount of hydrogen absorbed corresponded to 10.23 per cent of fumaric ester.

SURFACE TENSION OF SERUM. I.

Spontaneous Decrease of the Surface Tension of Serum.

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When a serum is exposed to the air, the surface energy of its free surface decreases progressively, according to a certain law, until after about 10 minutes a state of equilibrium is attained. The purpose of this paper is to report a study of this phenomenon, which has been overlooked so far on account of lack of proper apparatus for measuring surface tension.

The existence of a film at the surface of protein solutions has been discussed by Ramsden.[1] He pointed out the formation of semisolid or highly viscous films, and merely noted that "the matter which accumulates [in the surface layer] possesses the property of lowering the surface tension . . . . of the free surface of water."[1] The technique which he used did not allow him to proceed further in that line, and he neither observed nor measured any lowering in the surface tension of the serum itself, not did he notice the variations of the surface tension of water plus serum in function of the time. There is no doubt but that the existence of a more concentrated layer at the free surface or at all interfaces plays an important part in most biological phenomena. Whether this "film" is solid or liquid with simply higher viscosity will be discussed later. In order to prevent any misunderstanding, the word film will only be used to define a liquid film; the term "membrane" will be used with reference to a solid film.

I.

EXPERIMENTAL.

Over 3,000 measurements of surface tension were made. The instrument used was the tensiometer, previously described.[2] The

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[1] Ramsden, W., *Proc. Roy. Soc. London*, 1904, lxxii, 156.

[2] du Noüy, P. L., *J. Gen. Physiol.*, 1918–19, i, 521.

use of this apparatus is based upon the adhesion of a platinum ring to the surface of the liquid, and the stress necessary to tear off the film is measured by the torsion of a steel wire.   A measurement with this instrument requires approximately only 20 seconds, and the instrument always measures the tension of the same layer of liquid.

The ring method was adopted, after much experimenting, as the only reliable one for colloidal solutions, as well as the simplest, quickest, and most accurate.   This opinion is now shared by a large number of physicists, and particularly by Ferguson, who reported about twenty different techniques for measuring surface tension.[3]

The tensiometer[4] was used with an accurately calibrated platinum-iridium ring (circumference 4.00 cm.), and is reliable to ± 0.1 dyne. We believe that any data giving the second and third decimals are useless in general because, so far, none of the twenty odd methods in existence check each other, some differing by more than 5 dynes for the same substance at the same temperature, and because no theoretical reasons exist yet for adopting a standard value of surface tension for any substance.

All measurements were made between 23° and 25°C., room and liquid temperature.   All glasses, containers, and pipettes were boiled in cleaning solution (sulfuric acid and sodium dichromate), then rinsed in distilled water, and dried.   The free surface of the liquid was always 10 sq. cm. with a tolerance of ± 0.5 sq. cm.   The elapsed time between the pouring of the liquid and the measurement was very nearly constant, and equalled 15 seconds.   The ring was washed in distilled water and flamed after every measurement.   The same precautions were taken in collecting and preparing the serum.   Only clean glass was used.   No alcohol or ether was ever employed. When it occasionally happened that the edge of a tube had been touched with the fingers, a marked difference was observed in the readings.

The results are shown in Table I.

Text-figs. 1 and 2 show more clearly the progressive decrease of the surface tension.   It is quite remarkable that in 2 minutes the drop can be of such amplitude as 2 dynes.   In the three cases shown in

[3] Ferguson, A., *Science Progr. 20th Cent.*, 1914–15, ix, 428.
[4] Made by the Central Scientific Co. of Chicago.

Table I, the drop was very nearly 2.1 dynes. In 20 minutes, the surface tension was decreased by 4.1 and 3.3 dynes for the old dogs, and by 5.6 dynes for the very young dog. All three reached their equilibrium at about the same value.

If the serum is stirred at the end of the experiment, that is, when the surface tension seems to have attained a stable value, the homogeneity of the serum is momentarily reestablished, the surface tension rises

TABLE I.

Serum 1020 (Dog 12 Yrs. Old) (Text-Fig. 1).
Temperature 25°C.

Time.	0	2 m.*	6 m.	12 m.	20 m.	30 m.
Surface tension, *dynes*....................	59.6	57.5	56.9	56.1	55.5	55.5

Serum 1022 (Dog 1 Yr. Old) (Text-Fig. 1).
Temperature 23°C.

Time.	0	2 m.	6 m.	16 m.	25 m.	40 m.
Surface tension, *dynes*..........	61.1	59.0	57.8	56.0	55.6	55.6

Serum 1019 (Dog 12 Yrs. Old) (Text-Fig. 1).
Temperature 23°C.

Time.	0	2 m.	4 m.	8 m.	10 m.	20 m.	25 m.	30 m.	40 m.
Surface tension, *dynes*.	58.7	56.5	56.0	55.5	55.4	55.4	55.5	55.4	55.6

* In Tables I to X, the abbreviations h., m., and s. are used for hours, minutes, and seconds.
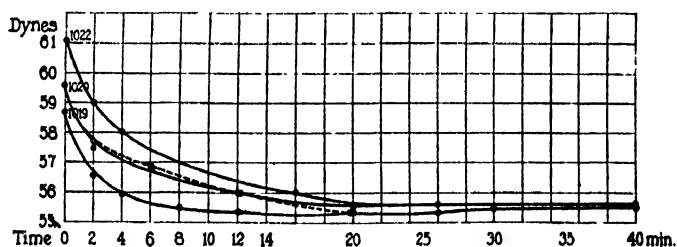
——— Observed curve.
------- Calculated curve.

TEXT-FIG. 1. Modification of the surface tension of serum in function of the time.
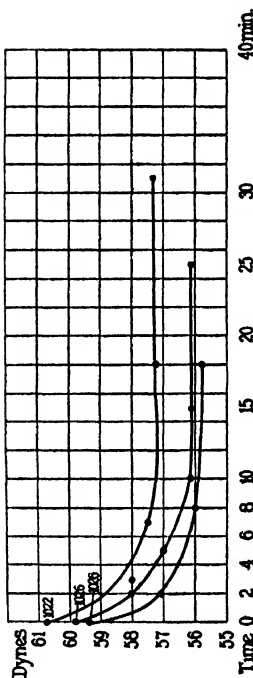
TEXT-FIG. 2. Modification of the surface tension of serum in function of the time. Two samples of the same serum (No. 1026) were kept 4 days in test-tubes, the first in the ice box (initial value 59.4 dynes), the second at room temperature (22°C.; initial value 59.8 d;nes).
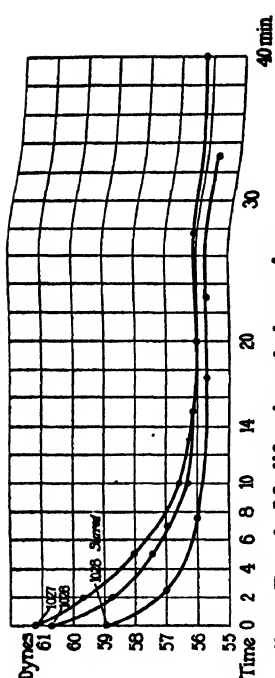
TEXT-FIG. 3. Modification of the surface tension of serum in function of the time.
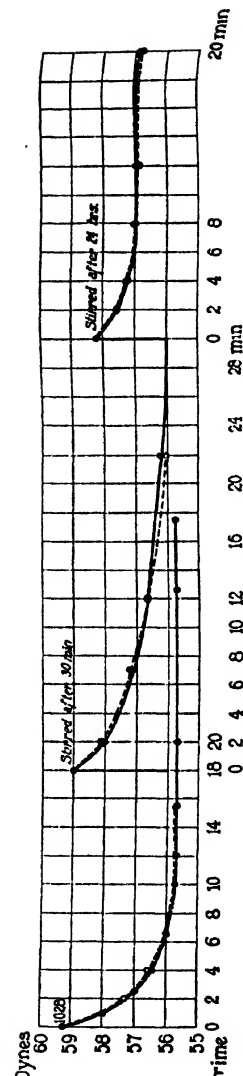
TEXT-FIG. 4. Effect of stirring on the surface tension of serum.

immediately, and the liquid recovers its property of forming another film. However, something is changed; the surface tension immediately after stirring does not reach its original value, and generally does not drop as much or as rapidly (Table II and Text-fig. 3). Text-fig. 4 shows plainly the change in the amplitude and in the rate of the phenomenon; the slope of the third curve (after 24 hours) is decidedly less marked; $\frac{d\gamma}{dt}$ decreases progressively.

TABLE II.

Serum 1027 (Dog 8 Yrs. Old).
Temperature 24°C.

Time.	0	2 m.	4 m.	8 m.	13 m.	23 m.	33 m.	4 h
Surface tension, *dynes*	59.8	58.5	57.9	57.1	56.5	56.5	56.3	56.3

Same serum stirred after 4 hrs., 59.0 dynes.

Serum 1028 (Dog 10 Yrs. Old) (Text-Figs. 3 and 4).
Temperature 24°C.

Time.	0	2 m.	7 m.	17 m.	27 m.	37 m.
Surface tension, *dynes*	59.3	57.1	56.0	55.7	55.7	55.7

Same serum stirred with a glass rod a few times, after 30 min., 59.0 dynes.

Time.	0	2 m.	4 m.	9 m.	14 m.	24 m.	50 m	2 h.	4 h.	24 h.
Surface tension, *dynes*...	59.0	58.0	58.0	57.1	56.9	56.0	56.0	55.4	56.0	55.4

Same serum stirred again, after 24 hrs., 58.2 dynes.

Time.	0	2 m.	4 m.	8 m.	20 m.	30 m.
Surface tension, *dynes*	58.2	57.6	57.3	57.0	56.9	56.9

The results were comparable in experiments on chicken serum (Table III).

An interesting fact, which may be considered as a good control, is that these two sera taken from similar chickens and showing at the beginning a difference of only 0.2 dyne in their surface tension have kept this difference practically constant during 50 hours, and after

TABLE III.

Serum 1053 (Chicken).
Temperature 25°C.   Oct. 11, 1921.

Time.	0	2 m.	4 m.	6 m.	11 m.	16 m.	26 m.	41 m.	56 m.
Surface tension, *dynes*.	62.0	61.2	61.0	61.0	60.7	60.0	58.0	57.0	57.0

Temperature 25°C.   Oct. 12, 1921.

Time.	20 h.	Stirred.	24 h.
Surface tension, *dynes*..	54.0	57.5	53.0

Temperature 25°C.   Oct. 13, 1921.

Time.	44 h.	Stirred.	47 h.	50 h.
Surface tension, *dynes*.................................	53.2	53.2	53.6	53.6

Serum 1054 (Chicken).
Temperature 25°C.

Time.	0	3 m.	6 m.	11 m.	20 m.	35 m.	1 h. 35 m.	16 h.
Surface tension, *dynes*.......	62.1	61.5	61.2	60.2	60.0	59.5	58.0	54.2

Serum stirred after 16 hrs., 58.0 dynes.

Time.	0	2 m.	12 m.	22 m.	38 m.	1 h. 10 m.	1 h. 40 m.	3 h. 55 m.	6 h.	7 h.
Surface tension, *dynes*..	58.0	58.0	57.1	56.9	56.0	55.0	54.6	53.2	54.6	54.6

Total time elapsed, 24 hrs.   On Oct. 13, 1921, after 40 hrs., 53.0 dynes. Stirred, 57.0 dynes.

Time.	0	3 h.	5 h. 30 m.
Surface tension, *dynes*......................................	57.0	54.0	53.9

being reactivated by stirring more than once. At the end of about 20 hours, the first was 54.0, and the second 54.2; at the end of 24 hours (after stirring), 53.0 and 53.2; at the end of 50 hours, 53.6 and 53.9, the difference here having become 0.3.
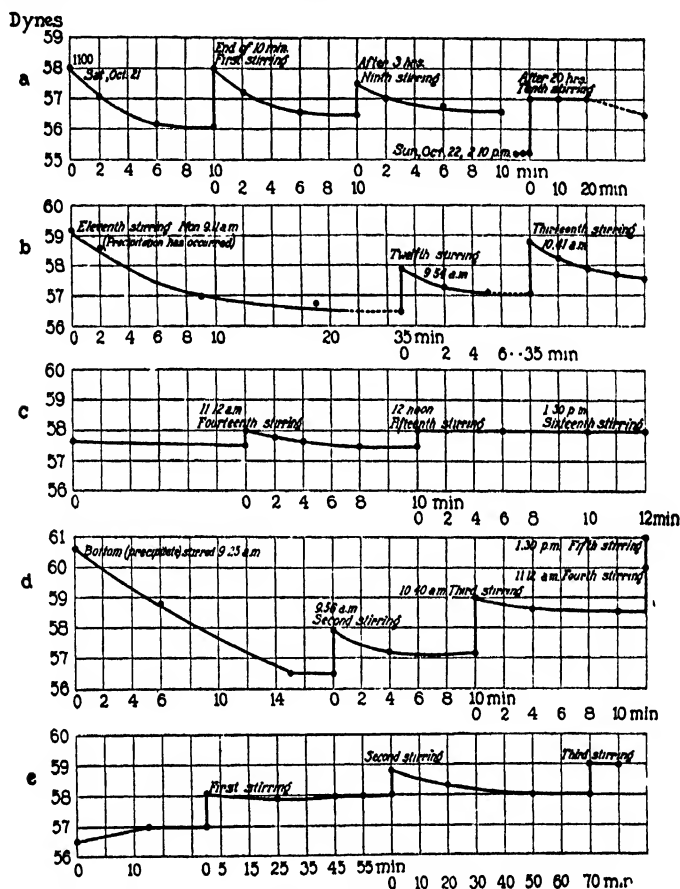
TEXT-FIG. 5, *a* to *e*. (*a* to *d*) Effect of stirring on the surface tension of serum. (*e*) Behavior of a control sample of the same serum, left unstirred during the 2 days, and stirred on Oct. 23, at 4 p.m., for the first time (precipitate at the bottom). Original value on Oct. 21, 56.5 dynes.

The rise in surface tension which follows the redissolution of the liquid surface layer by stirring is a general phenomenon with all clear, fresh sera. The progressive decrease in the activity, which can be compared graphically to a damping effect, seems constant also (Text-fig. 5, *a* to *e*). But it often happens that after 24 hours the property

TABLE IV.

Serum 111 (Rabbit).
Temperature 23°C.

Time.	0	2 m.	15 m.	25 m.	55 m.	1 h. 35 m.
Surface tension, *dynes*	57.5	56.5	55.5	55.4	54.7	54.6

Serum stirred after 1 hr. and 35 min., 56.9 dynes.

Time.	0	2 m.	6 m.	11 m.	26 m.	44 m.
Surface tension, *dynes*	56.9	56.1	55.5	54.6	55.0	54.7

After 2 hrs. and 20 min., the surface tension was 54.7 dynes.   The next morning, after 18 hrs. and 20 min., it was 53.6 dynes.   Stirred, it rose to 55.1 dynes.

Time.	0	2 m.	10 m.	1 h. 15 m.	2 h. 15 m.	2 h. 20 m.
Surface tension, *dynes*	55.1	55.0	55.0	55.1	55.0	55.0

Serum stirred at beginning of experiment, 55.1 dynes.   Stirred after 2 hrs. and 20 min., 55.0 dynes.

Serum 1060 (Dog).
Temperature 23°C.

Time.	0	2 m.	6 m.	13 m.	23 m.	38 m.	2 h. 8 m.	3 h.	3 h. 15 m.
Surface tension, *dynes*	59.3	57.5	56.8	56.0	55.5	55.4	56.0	56.0	56.0

Serum stirred after 3 hrs. and 15 min., 58.0 dynes.

Time.	0	2 m.	5 m.	10 m.	40 m
Surface tension, *dynes*	58.0	57.1	57.0	56.7	56.0

Time.	0	2 m.	6 m.	14 m.	19 m.	50 m.	1 h. 20 m.
Surface tension, *dynes*	56.2	55.6	55.6	55.6	55.6	55.6	56.0

After 18 hrs. the surface tension was 54.2 dynes; stirred, 56.0 dynes; stirred again after 19 hrs. and 20 min., 56.0 dynes.

is completely lost. The serum is inactivated from this standpoint and its surface tension remains constant (Table IV). As will be observed, the decrease still continued after the 4th hour, and over night it dropped from 56 to 54.2 dynes. At the end of the experiment, the serum became too viscous and had to be discarded.

Action of Heat.

Time is not the only factor which inhibits this activity of the serum. Heat acts in the same way; it may not act definitively by destroying

TABLE V.

Serum 1020 (Dog; Clear, Viscous).
Temperature 25°C. Heated for 20 Hrs. at 55°C.

Time.	0	2 m.	8 m.	18 m.	33 m.
Surface tension, *dynes*	56.0	56.0	56.0	55.7	55.8

Serum 1021 (Dog; Cloudy).
Temperature 24°C. Heated for 20 Hrs. at 55°C.

Time.	0	2 m.	5 m.	11 m.	20 m.
Surface tension, *dynes*	57.5	57.1	57.3	57.3	57.3

Serum 1022, Control (Dog; Clear).
Temperature 24°C. Unheated.

Time.	0	3 m.	6 m.	11 m.	17 m.	31 m.
Surface tension, *dynes*	60.8	58.0	57.5	57.4	57.1	57.3
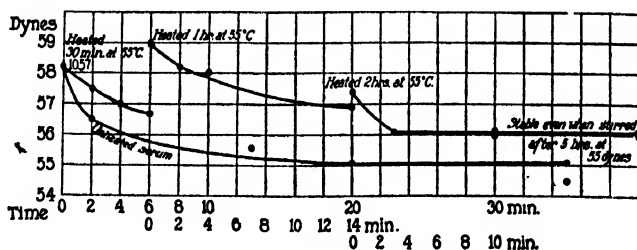
TEXT-FIG. 6. Action of heat on the surface tension of serum.

the substance, but the behavior of the serum is modified profoundly at the start, as will be seen in Table V.

TABLE VI.
Serum 1057 (Dog; Clear) (Text-Fig. 6).
Temperature 24°C.   Heated for 30 Min. at 55°C.

Time.	0	2 m.	4 m.	8 m.
Surface tension, *dynes*................................	58.2	57.5	57.0	56.7

Temperature 24°C.   Heated for 1 Hr. at 55°C.

Time.	0	2 m.	4 m.	14 m.
Surface tension, *dynes*................................	59.0	58.2	58.0	56.9

Temperature 24°C.   Heated for 2 Hrs. at 55°C.

Time.	0	3 m.	5 m.	10 m.	25 m.	55 m.
Surface tension, *dynes*....................	57.4	56.1	56.1	56.1	56.1	56.1

Serum stirred after 55 min.; surface tension 61.0 dynes.

The Same Sample, after 20 Hrs.   Temperature 24°C.

Time.	0	2 m	9 m.	19 m.	34 m.
Surface tension, *dynes*.........................	61.0	60.7	60.0	59.5	59.2

Temperature 24°C.   Heated for 3 Hrs. at 55°C.

Time.	0	10 m.	45 m.	1 h. 15 m.	2 h. 15 m.	3 h.
Surface tension, *dynes*....................	59.3	57.0	57.0	57.4	57.5	57.4

Apparently, at least 5 hours heating at 55°C. were necessary to inactivate the sera completely, although after 2 hours the activity was reduced, as shown by Table VI and Text-fig. 6.   In this case, the control itself was inactivated after 24 hours without heating.

Effect of Precipitation of Insoluble Substances in the Serum.

All the sera in which a precipitation of insoluble substances had occurred, in the form of a coarse colloidal suspension showing a tendency to settle out spontaneously after a few hours standing, were devoid of any activity (Table VII). But if the precipitate was allowed to settle, after the liquid had been stirred, the surface tension was decreased.

TABLE VII.

Serum 1059 (Dog; Cloudy; Precipitate in Suspension).
Temperature 23°C.

Time.	0	2 m.	5 m.	32 m.
Surface tension, *dynes*.................................	53.8	53.8	53.6	53.6

When a serum is allowed to dry entirely and is redissolved in plain water, it still manifests the same activity, although to a smaller extent (Table VIII and Text-fig. 7).

TABLE VIII.

Serum 1027 (Dog).   Dried and Dissolved in Water (Text-Fig. 7).
Temperature 24°C.

Time.	0	2 m.	5 m.	12 m.	27 m.	42 m.	1 h. 27 m.	2 h. 50 m.
Surface tension, *dynes*.......	58.0	57.2	57.2	56.9	56.1	56.2	56.2	56.1

Serum 1027, Control.
Kept at Room Temperature for 48 Hrs. (23°C.).

Time.	0	2 m.	4 m.	7 m.	12 m.	22 m.	45 m.	1 h. 15 m.	1 h. 30 m.	2 h.	2 h. 15 m.	2 h. 30 m.	2 h. 45 m.	3 h. 45 m.	8 h.
Surface tension, *dynes*..	60.0	57.5	57.0	56.1	55.9	55.5	55.6	55.7	56.0	56.1	56.2	56.5	56.2	56.5	56.5

Same serum stirred, 58.0 dynes.

Great care is necessary when taking the measurements, for the slightest agitation of the surface causes the film to dissolve, with·a

consequent rise in the surface tension.   The first series of measurements was more carelessly done, and gave results which did not check with the later ones.   We had to make a set of very careful measurements in which the liquid was absolutely undisturbed, to
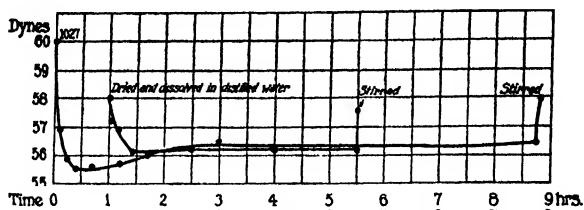
TEXT-FIG. 7. Surface tension of fresh serum, and of the same sample dried and dissolved in water.

find out the reason for such unaccountable facts as a spontaneous rise after a drop, such as that shown in Text-fig. 8.

Text-fig. 5, *e* illustrates the damping effect again, and also shows that the progressive inactivation is mainly function of the time and
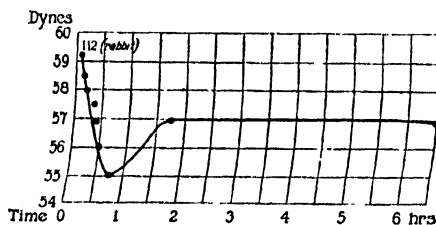
TEXT-FIG. 8. Spontaneous rise in surface tension of serum after a fall.   The rise after 40 minutes and after 6½ hours was due to the careless handling of the watch-glass containing the serum.

is but little affected by stirring.   It illustrates also the progressive increase—reverse phenomenon—which takes place after each stirring, when precipitation has occurred in the serum.   This is probably due, as pointed out elsewhere, to the formation of a membrane or semisolid film in the surface layer (see also Text-fig. 5, *d*).

II.

DISCUSSION.

The hypothesis of surface coagulation, or the formation of an homogeneous solid film, does not seem to us to account for any of the phenomena studied. First, there is no sign, optically, of such a film. Moreover, a solid film would increase, rather than decrease, the surface tension; and finally, it would not account for the damping of the phenomenon, in function of the time. Besides, by very light stirring the tension rises again, and most of the sera experimented upon remained perfectly clear, although their viscosity increased, even after 48 hours.

An objection may be made that temperature and evaporation (concentration) play a part in this phenomenon. But a decrease in temperature, which actually occurs as soon as a liquid is spread over a larger surface, would mean an increase in surface tension, whereas we observe the opposite. Concentration following evaporation requires a good deal of time; after 20 minutes, samples were tested by measurements of their cryoscopic point and did not show any marked concentration. As the phenomenon usually takes place in less than 15 minutes, it may be concluded that it can only be affected by the change in the concentration of the surface layer. With highly concentrated sera, evaporated *in vacuo* and reduced to one-third of their original volume, the figures were practically the same.

A few hypotheses are hence possible. Either the drop in surface tension is due to a chemical change and adsorption of the resulting substances in the surface layer, or to an adsorption of bodies generated or contained in the bulk, or else to modifications in the arrangement of the group molecules in the surface layer, or possibly to a combination of these causes. Without making any attempt to discuss the structure of the surface layer, the facts may be accounted for in the case of the first hypothesis by assuming that the huge, fragile protein molecules begin to disintegrate and yield substances acting on surface tension as soon as they are no longer entirely surrounded by others in the liquid, on account of the change in potential energy. In the process of breaking down, they may also liberate progressively

other molecules, the accumulation of which would inhibit the phenom-
enon by concentrating in the surface layer and decreasing definitively
the surface tension.

But it is not necessary to assume that the substances which lower
the surface tension are produced in the surface layer. Indeed, the
reaction occurs as if these substances came slowly from the bulk of
the liquid. An exceedingly slight change in the concentration of
certain substances in the serum could explain the facts. But there
must be some chemical change to account for the damping effect, and
for the inactivation, unless it is attributed to adsorption by colloidal
particles. This being plainly a case of adsorption, let us consider
what the differences are between this phenomenon and the ordinary
phenomenon of adsorption.

In the ordinary case of adsorption, when a substance lowering the
surface tension is added to the liquid the value for the surface tension
corresponds to the concentration. Equilibrium is usually rapidly
established with respect to the quantity adsorbed in the surface layer
in excess of the quantity in the bulk, and the surface tension may reach
a very low value.

In the present case, on the contrary, all constituents are coexistent,
so that, at first sight, the concentration seems constant, as the sub-
stance lowering the surface tension exists normally in the serum. But
either a reaction or a migration takes place in function of the time,
which progressively changes the ratio of concentrations between the
bulk and the surface layer. From 10 to 20 minutes are required to
reach the equilibrium, which takes place generally between the limits
53 and 56 dynes, never below 53.

In the ordinary case of adsorption, the time taken for attainment
of equilibrium is shortened by shaking. In the case of serum, con-
versely, shaking stops the process, destroys the film, and brings back
the original surface tension, almost to its former value. The time
required for attainment of equilibrium is not shortened, but
lengthened.

Why substances lowering surface tension remain adsorbed in the
surface layer, instead of remaining in the bulk as do the bodies which
raise surface tension, is theoretically accounted for by the dynamical

theory of solutions, as developed mainly by Gibbs[5] and Thomson.[6] It may be of interest to the reader to recall this theory briefly.

The most stable arrangement of any solution must be one accompanied by minimal surface tension. This follows from the simple study of Gibbs' equation:

(1)
$$c\frac{d\gamma}{dc} = -u\frac{dp}{dc}$$

in which u is the quantity adsorbed, p the osmotic pressure, c the concentration, and γ the surface tension. For dilute solutions, the osmotic pressure is given by the equation

(2)
$$p = RT$$

in which R is the constant of gases and T the absolute temperature. Therefore,

(3)
$$dp = RTdc$$

By introducing this value in (1),

(4)
$$u = -\frac{c}{RT} \cdot \frac{d\gamma}{dc}$$

As stated above, u is precisely the adsorption; that is, the difference between the concentrations of the surface layer and the bulk. It is called positive when the concentration in the surface layer is greater, and negative when it is smaller than in the bulk. $\frac{d\gamma}{dc}$ is the differential coefficient of the function connecting surface tension and concentration, and is positive if γ and c increase together and negative if γ decreases with increasing c. Therefore, the whole product on the right hand of the equation will be *negative* in the first case (when the solute *increases* surface tension) and *positive* in the second (when the solute *reduces* surface tension) This means a lower concentration in the surface layer (smaller amount adsorbed, u) in the former case, and a higher concentration in the surface layer in the latter; R, T,

---

[5] Gibbs, W., *Tr. Conn. Acad*, 1878, iii, 380, Scientific papers, London, 1906.

[6] Thomson, J. J, Applications of dynamics to physics and chemistry, London, 1888, 191.

and c being necessarily positive, the sign of u depends only on that of $\dfrac{d\gamma}{dc}$.

This explains why Gibbs' law is often given under the following form. A small amount of dissolved substance may reduce the surface tension considerably, but can only increase it slightly.

It is possible to account not only for the decrease in surface tension represented by the first curves, but also for the damping of the phenomenon, by assuming that some kind of chemical or physicochemical change is undergone by the substances in solution in the serum, in function of the time. The action of these substances, which would accumulate slowly, would be superimposed on that of the bodies acting normally, and would progressively lower the initial value of the surface tension. The presence of these new substances, whatever they are, would forcibly change the physicochemical qualities of the serum. Hence the curve representing this second stage of the phenomenon is slightly modified. The rate of the lowering of surface tension is slower, owing probably to the increased viscosity of the serum with time. When ten stirrings are made within 100 minutes, followed by a lowering of surface tension, the change in the curves, that is, in the rate of decrease, is not very different from that which would have been obtained had the serum been stirred only once at the end of the 100 minutes. Time acts more efficiently than stirring.

When a serum becomes old, precipitation occurs in the surface layer where the concentration is higher, and insoluble substances are made out of soluble ones. These ultramicroscopic particles agglomerate little by little into larger ones, until they are so heavy that they precipitate. They have a tendency to transform the surface layer into a semisolid, semiliquid layer with higher surface tension (Experiment 1059, Table VII). In order to prevent confusion we shall refer hereafter to the reaction which takes place for the first time, as soon as the serum is exposed to the air, as the first reaction. After the first stirring, it will be the second reaction, and so on. There is no doubt that one reaction takes place as soon as the blood is removed from the body, and another every time the serum is poured from one tube to another. But in such cases, the surface exposed is small and when not moved is protected by the first film formed. Besides, we

must arbitrarily choose a starting point, and mathematical study of the phenomenon shows that our assumption is probably correct.

III.

Mathematical Expression of the Phenomenon.

An attempt was made to establish a general equation on a purely empirical basis, which would express the phenomenon in all cases, before and after stirring, with only one coefficient. We found that the exponential formula

$$(5) \qquad \gamma = \gamma_0 e^{-Kt^{\frac{1}{2}}}$$

expressed the facts very closely (in the case of dog and rabbit serum), until the phenomenon became asymptotic to a parallel to the axis of time, the ordinate of which is generally around 55.5 dynes. After 10 to 20 minutes, the curve becomes parallel to the axis of time and the phenomenon stops. This fact may be expressed, if required, simply by the addition of a linear term to formula (5). We have, then,

$$(6) \qquad \gamma = \gamma_0 e^{-Kt^{\frac{1}{2}}} + At - B$$

t being the time, γ the surface tension at the time t, γ_0 the surface tension at the beginning of the experiment, K, A, and B three constants. In fact, the constants A and B do not modify the law, and the corrective term is only required for the first reaction. After stirring, the first simple formula (5) suffices to account for the facts with close agreement practically to the point when equilibrium is attained. Indeed, the phenomenon is characterized by only one constant, K, generally equal to 0.01, for average old dogs; B is equal to 1.6 (in the example chosen—Serum 1020, Table X), and A to 0.1.

There may be other equations by means of which this law can be expressed, as always occur in cases of logarithmic curves. But the advantage of this exponential form is that it requires only one constant, the meaning of which is clearer, and that it is short and, as is well known, very easy to calculate under the form

$$(7) \qquad \text{Log } \gamma = \text{Log } \gamma_0 - K\sqrt{t}$$

Tables IX and X show the agreement between observed and calculated figures.   The two curves in Table X were calculated with the simple formula (5).   It will be noticed that the constant K decreased rapidly after each stirring, indicating that the serum becomes saturated, or inactivated, after a certain time.

<div align="center">

TABLE IX.

Serum 1028 (Dog).
First Reaction.
$K = 0.01$

</div>

Time.	0	2 m.	4 m.	6 m. 5 s.	16 m.	20 m.	40 m.
Surface tension observed, *dynes*	59 3	57.2	56.5	56.0	55.7	55 7	55.7
" " calculated, *dynes*		57.3	56.6	56.0	55.7	55.7	55.7

These figures are obtained from the 16th min. on, with the complete formula (6). $C = 0.17$ and $A = 1$.

<div align="center">

Second Reaction; Stirred after 30 Min. (Text-Fig. 4).
$K = 0.005$

</div>

Time.	0	2 m.	7 m.	12 m.	22 m.
Surface tension observed, *dynes*	59.0	58.0	57.1	56.7	56.1
" " calculated, *dynes*		58.1	57.2	56.7	56.0

<div align="center">

Third Reaction; Stirred after 24 Hrs. (Text-Fig. 4).
$K = 0.00325$

</div>

Time.	0	2 m.	4 m.	8 m.	12 m.
Surface tension observed, *dynes*	58.2	57.6	57.3	57.0	56.9
" " calculated, *dynes*		57.6	57.3	57.0	56.8

The progressive decrease in surface tension after each stirring is shown again in Text-fig. 5, *a*.   As stated before, this phenomenon seems to happen only when there is no precipitation in the liquid; as soon as a certain amount of precipitation has taken place, the surface tension, after stirring, rises higher than its former values.

It may seem very premature to try to apply any theoretical formula to this phenomenon, considering the enormous complexity of the

liquid studied, of whose constitution we are so ignorant. However, an interesting fact leads us to believe that it may some day be done successfully. As already noted this phenomenon is one of adsorption in the surface layer. Mere reasoning makes this clear. But a comparison of one of our curves with a curve obtained by Lewis[7] for the adsorption of sodium glycocholate by a surface of paraffin oil shows a

TABLE X.

Serum 1020 (Dog under 1 Yr. of Age).
$K = 0.0065$

Time.	0	2 m.	6 m.	12 m.	20 m.	25 m.
Surface tension observed, *dynes*............	59.6	57.5	56.9	56.1	55.5	55.5
" " calculated, *dynes*..........		57.7	56.9	56.0	55.3	55.3

In this case, the coefficient is much smaller.

Serum 1022 (Old Dog).
$K = 0.01$

Time.	0	2 m.	6 m.	16 m.	25 m.
Surface tension observed, *dynes*.................	61.1	59.0	57.8	56.0	55.6
" " calculated, *dynes*................		59.0	57.75	55.8	

remarkable identity despite the entirely different conditions (Text-figs. 9 and 10). A few formulas have been given for expressing the phenomenon of adsorption in function of the quantity adsorbed and of the concentration. It is clear that they could not be used in our case. Among these we may mention the formula quoted by Duclaux[8] and that of Freundlich,[9] inaccurate at higher concentrations as shown by Schmidt,[10] who gives a rather complicated exponential formula[11]

[7] Lewis, W. C. McC., *Proc. Physic. Soc.*, 1909, xxi, 150; *Phil. Mag.*, 1909, xvii, 466; *Z. Chem. u. Indust. Kolloide*, 1909, v, 91; also cited by Willows, R. S., and Hatschek, E., Surface tension and surface energy, and their influence on chemical phenomena, Philadelphia, 1915, 41.

[8] Duclaux, J., Les colloïdes, Paris, 1920, 148.

[9] Freundlich, H., Kapillarchemie: Eine Darstellung der Chemie der Kolloide und verwandter Gebiete, Leipsic, 1909, 146.

[10] Schmidt, G. C., *Z. physik. Chem.*, 1910, lxxiv, 699.

[11] Schmidt, G. C., *Z. physik. Chem.*, 1911, lxxvii, 641.

with two constants. This equation applies well to the adsorption of liquids by solids, and Arrhenius[12] has pointed out that it could be
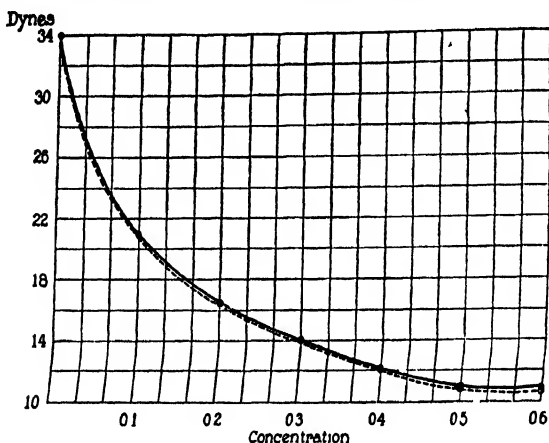
TEXT-FIG. 9. Action on surface tension of adsorption of sodium glycocholate by paraffin oil (Lewis; adsorption isotherm).
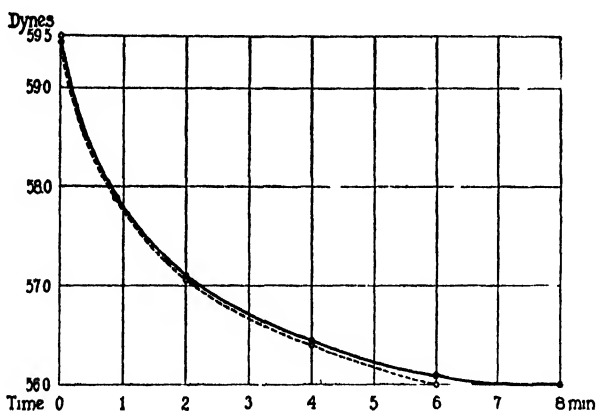
TEXT-FIG. 10. Action on surface tension of adsorption in the surface layer of serum.

simplified. A formula in function of surface tension was given by Sentis[13] in 1897, and another by Valson in 1872.[14] The last two only

[12] Arrhenius, S. A., Conférences sur quelques thèmes choisis de la chimie physique, Paris, 1912, 31.

[13] Sentis, Recueil de constantes physiques, Paris, 1913, 122.

[14] Valson, cited by Morgan, J. L. R., and McKirahan, W. W., *J. Am. Chem. Soc.*, 1913, xxxv, 1759.

apply to inorganic salts, which raise the surface tension and therefore are not adsorbed in the surface layer. Of course, the curve of Lewis is not drawn in function of the time, but in function of the concentration of the solution. In our case, the concentration is constant, but we can admit that the relative concentration is proportional to the time, since the solute which lowers the surface tension is either formed in the free surface layer as soon as it exists, or travels through the bulk to it, in function of the time.

The perfect identity of the curves is emphasized by the fact that the new equation (5), which we proposed for the phenomenon studied in this paper, applies remarkably well to that of Lewis (Table XI). This gives a greater character of generality to this formula, which

TABLE XI.

Agreement between Figures Observed and Calculated for the Lowering of Surface Tension at the Interface between a Solution of Sodium Glycocholate and Paraffin Oil, in Function of the Concentration (Text-Fig. 9).
$$K = 0\ 222$$

Concentration, *per cent*	0	0 1	0 2	0 3	0 4	0 5	0 6
Surface tension observed (Lewis), *dynes*	34 0	21 0	16 5	14 0	12 2	11 0	10 9
Surface tension calculated $(\gamma = \gamma_0\, e^{-Kc^{\frac{1}{2}}})$, *dynes* ...		20 2	16 5	14 0	12 2	10 9	9 7

may be applied to the phenomenon studied by Traube,[15] Lewis,[7] Donnan and Barker,[16] Langmuir,[17] and others, simply by changing t to c; namely,

(8)
$$\gamma = \gamma_0 e^{-Kc^{\frac{1}{3}}}$$

Toward the end of the curve, it becomes parallel to the abscissa of ordinate 10, as a state of equilibrium is attained, and, for the same reasons as expressed above, the calculated data deviate from those observed (Text-fig. 9).

[15] Traube, J., *Kolloidchem. Beihefte*, 1911–12, iii, 237.

[16] Donnan, F. G., and Barker, J. T., *Proc. Roy. Soc. London, Series A*, 1911, lxxxv, 557.

[17] Langmuir, I., *J. Am. Chem. Sc.*, 1917, xxxix, 1848.

Besides, as the measurements of Lewis were made with the drop weight method, it shows that the method used in our experiments on colloidal substances is as reliable as the most accurate and time-consuming methods used by Lewis.

To give an idea of the order of magnitude of the excess of concentration in the surface layer, since we cannot measure u directly, we may use Gibbs' formula tentatively, without laying emphasis upon the figures given here. Assuming the total concentration in the bulk of a serum to be 9.9 per cent after 1 minute (electrolytes and nonelectrolytes), and referring to the formula

$$(4) \qquad\qquad u = -\frac{c}{RT} \cdot \frac{d\gamma}{dc}$$

we have the values

$$c = 9.9; \ R = 83.2 \times 10^6; \ T = 293°; \frac{d\gamma}{dc} = \frac{d\gamma}{dt} = 2$$

$\dfrac{d\gamma}{dc}$ = the tangent of the angle of the tangent to the point of abscissa 1 with the axis of the abscissæ.

$$u = 0.81 \times 10^{-7} \text{ gm. per sq. cm.}$$

The free surface being about 10 sq. cm., it gives a total amount, roughly, of $\dfrac{1}{1,000,000}$ gm. of substance adsorbed in the surface layer; but as we deal with colloidal solutions, this figure is almost certainly wrong.

As a point of comparison, it may be said that for sodium glycocholate Lewis found experimentally that $u = 3.6 \times 10^{-6}$ instead of about 1.0×10^{-7}, the theoretical value; and Donnan and Barker, using nonylic acid as solute, obtained for u, 0.95×10^{-7}, instead of 0.55×10^{-7}, calculated.

We must not forget that the theoretical values of u (quantity adsorbed) in certain cases were twenty to thirty times smaller than the experimental values. This may be due partly to great difficulties in the technique. The discrepancy is still more marked for colloidal substances. Langmuir[17] has insisted upon the differences existing between theoretical and experimental data.

Further experiments are now being carried out on this subject.

IV.

CONCLUSIONS.

1. Over 3,000 measurements of surface tension of sera have been made with the ring method, and they have yielded a new phenomenon, the spontaneous and rapid decrease of the surface tension of a serum in function of the time.

2. Generally, after 10 minutes the surface tension reaches a value which is practically constant. At least, the decrease is very much slower. After stirring, a rise occurs and a similar phenomenon takes place; but stability is not obtained as rapidly, requiring about 25 minutes. By stirring again, the same thing happens repeatedly, the slope of the curve being less marked each time, the rise in surface tension being slightly below each previous value, and the phenomenon undergoing a sort of damping.

3. An equation was established which expresses the experimental facts with an accuracy of about 0.2 per cent. It applies to the whole phenomenon, before and after stirring. It has only one characteristic constant,

$$\gamma = \gamma_0 e^{-Kt^{\frac{1}{3}}}$$

This formula, by simply changing t to c (concentration), expresses satisfactorily in general the phenomenon of adsorption in the surface layer; that is, the decrease in surface tension in function of the concentration.

4. Prolonged heat, at 55°C., and time seem to inhibit this phenomenon.

5. When precipitation occurs in a serum, the bottom of the liquid, which contains the precipitate, has the highest surface tension. When stirred, the surface tension rises a little every time. The upper part, clear, with lower surface tension, shows the reverse phenomenon; after every stirring, the surface tension becomes a little lower.

SURFACE TENSION OF SERUM.

II. ACTION OF TIME ON THE SURFACE TENSION OF SERUM SOLUTIONS.

BY P. LECOMTE du NOÜY, Sc.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 58 TO 61.

I.

INTRODUCTION.

It has been shown in a previous paper[1] that the surface tension of serum decreased rapidly, as soon as the serum was exposed to the air. This phenomenon is due to the adsorption, in the surface layer, of substances which either travel slowly in the liquid, or are formed in the surface layer, or else to modifications in the arrangements of the group molecules in the surface layer, and possibly to a combination of these causes. In order to study these substances and to obtain an idea of the order of magnitude of their power of lowering the surface tension of water in function of time, it was necessary to measure the surface tension of the same samples of solutions of serum at different intervals, ranging from 2 minutes to 24 hours.

II.

EXPERIMENTAL.

1. Decrease of Surface Tension of Serum Solutions in Function of Time.—Solutions of fresh serum were made in saline solution (NaCl 0.9 per cent). The surface tension of the same layer of liquid was measured by means of du Noüy's tensiometer, according to the technique previously described.[1] In order to prevent any jarring of the samples studied, a turntable was made, supported on a ball thrust bearing, on which ten watch-glasses were placed in a circle. By

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[1] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.

turning the table carefully, the watch-glasses were brought succes-
sively under the ring, and then raised smoothly by means of the screw
of the instrument until the contact was established. The results
obtained are shown in Table I.

Higher dilutions were prepared and tested after 1, 2, 20, and 23
hours, with the results shown in Tables II and III.

This phenomenon is illustrated by Text-fig. 1. In the charts, the
value of the surface tension is taken after 2 hours arbitrarily, but the
maximum drop occurs in the first 30 minutes (Text-fig. 2). The lapse
of 2 hours was chosen because after that period of time, the drop is
usually very slow. Text-figs. 3 and 4 show that between 1 and 2
hours the drop is small.

The drop in surface tension may be expressed in two different ways
according to whether one chooses to take into account either the initial
value of the surface tension as measured after a few seconds, or else
to assume that the drop is so rapid at the beginning that a difference
of 1 second or less is sufficient to affect the initial reading. In the
latter case, the initial value may be taken as equal to 76 dynes,
roughly, and the differences expressed thus:

$$- d\gamma = 76.0 - \gamma$$

The initial values and the values after 2 hours are shown in Text-
figs. 3 to 9. The drop, $- d\gamma = \gamma_0 - \gamma$, is expressed in Text-figs. 10
to 15, and the drop, $- d\gamma = 76.0 - \gamma$, in Text-fig. 16.

2. *Comparison with the Behavior of Some Organic Salts.*—It may be
of interest to compare the action of serum with that of substances
having a strong effect on the surface tension of water, such as sodium
oleate, sodium glycocholate, and saponin, at similar dilutions in the
same saline solution (Table IV). The phenomenon is illustrated in
Text-figs. 17 to 19. Comparison with Text-figs. 3 to 9 shows the
likeness to the behavior of serum, although the initial value is much
higher in the latter case. This fact is probably due to substances
which have some kind of buffing action, and oppose the lowering of
surface tension of serum. The substances are particularly active
when a foreign body, such as oleate or glycocholate, is added to the
serum. This phenomenon will be studied in another paper.[2]

[2] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv (in press).

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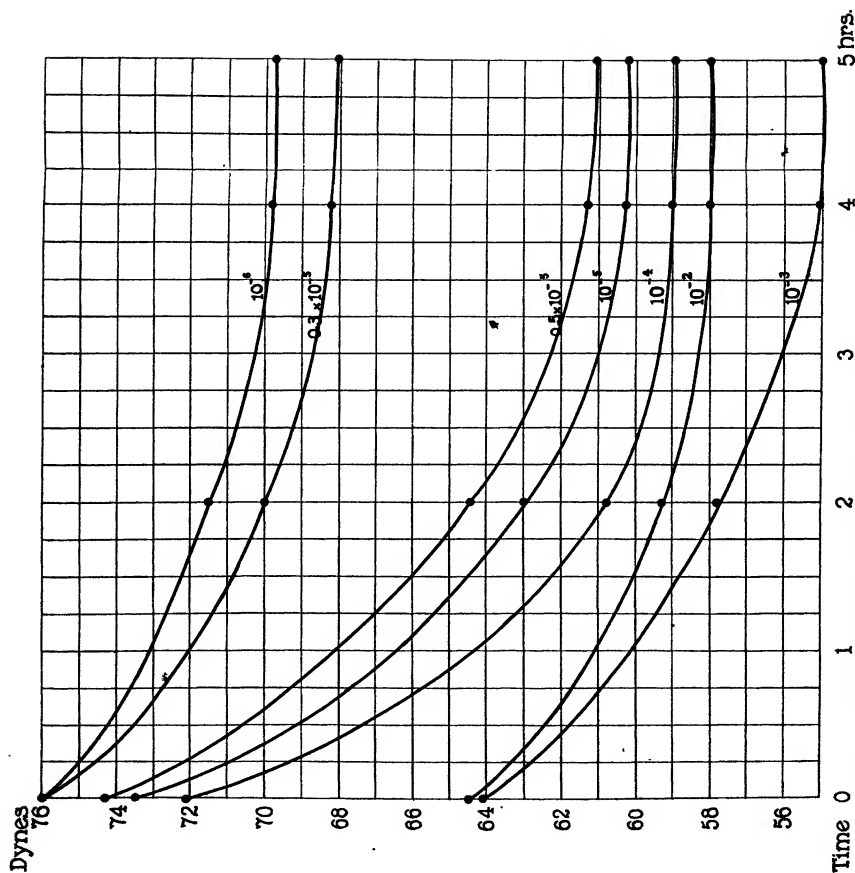
TEXT-FIG. 1. Values of the surface tension of serum solutions in function of the time.   Serum 118 (rabbit).

## TABLE I.

Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.

Fresh Rabbit Serum, No. 114 (Text-Fig. 2).
Temperature 22° C.

Time..................	0	2 min.	6 min.	10 min.	30 min.	2 hrs.	Drop in 2 hrs.
Pure.	65.5*	64.5	64.0	64.0		63.0	2.5
Dilution.							
1:100	69.0	67.0			64.0	59.3	9.7
1:1,000	67.0	65.0			62.5	53.0	14.0
1:5,000	71.6	69.5			58.0	52.6	19.0
1:10,000	72.0	68.1	64.6	63.0	60.0	56.0	16.0
1:20,000	75.5	73.0			67.5	61.0	14.5
1:30,000	75.0	74.0			67.0	63.0	12.0
1:40,000	72.2	70.0			67.2	62.0	10.0
1:50,000	75.8	75.8			70.0	65.6	10.2
1:100,000	68.0	68.0			62.0	56.9	11.1
1:200,000	73.5	73.0			69.0	62.0	10.5

* In all the tables the determinations are given in dynes.

TABLE II.

Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.

Fresh Rabbit Serum, No. 114.
Temperature 22° C.

Time..................	0	1 hr.	2 hrs.	20 hrs.	23 hrs.	Drop in 23 hrs.
Dilution.						
1:400,000	67.0	76.2	67.0		64.0	12.0
1:800,000	76.2	73.2	70.7		67.0	9.2
1:1,000,000	76.0		69.5	70.1		6.0
1:3,000,000	73.5	71.5		69.0		4.5
1:5,000,000	72.1			70.1		2.1
Control.						
Pure saline solution, 0.9 per cent...........	75.3	.			73.5	1.8
2nd sample.	76.5				75.6	1.5
3rd " covered...	76.2				76.2	0 '

TABLE III.

Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.

Fresh Rabbit Serum, No. 116 (Text-Fig. 3).

Temperature 20° C.

Dilution	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	68.3	71.0	73.5	76.0	76.5
After 2 hrs	62.0	61.8	62.0	65.9	72.0
" 5 "	58.9	57.0	57.5	64.0	71.5

Fresh Rabbit Serum, No. 117 (Text-Fig. 5).

Temperature 20° C.

Dilution	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	66.1	66.5	72.9	76.0	76.3
After 2 hrs	63.0	63.0	64.5	68.2	73.2
" 5 "	60.0	59.2	59.0	63.0	71.4

Fresh Dog Serum, No. 1 (Text-Fig. 6).

Temperature 20° C.

Dilution	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	63.5	66.0	73.0	75.0	76.0
After 3 hrs	58.0	57.3	59.0	62.5	70.0

Fresh Human Serum (Text-Fig. 8).

Temperature 22° C.

Dilution	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	61.1	65.0	72.0	76.0	76.0
After 2 hrs	55.5	57.0	58.0	61.9	73.0

Text-Fig. 2. Values of the surface tension of a serum solution in the first 40 minutes. Dilution 1:10,000. Serum 114 (rabbit)
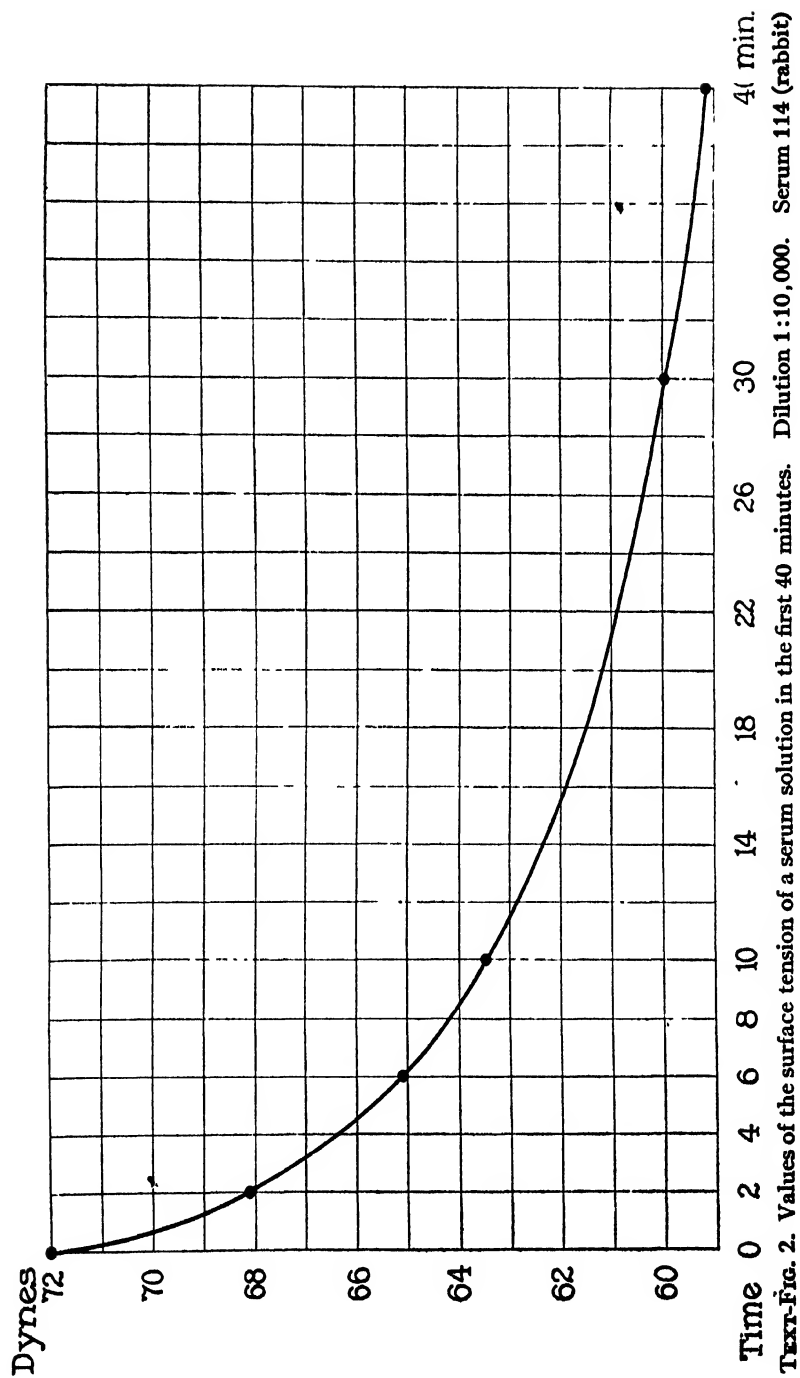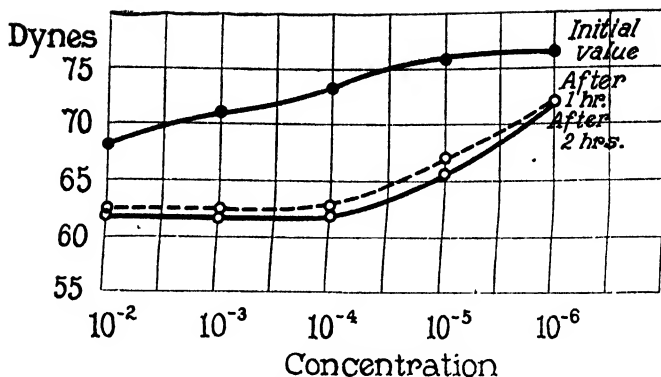
TEXT-FIG. 3. Values of the surface tension of serum solutions in function of the time. Serum 116 (rabbit).
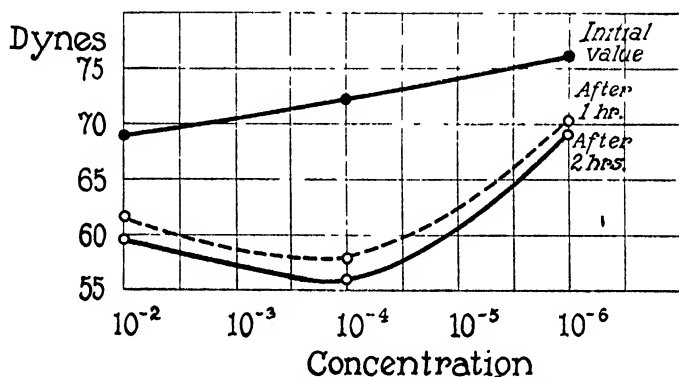
TEXT-FIG. 4. Values of the surface tension of serum solutions in function of the time. Serum 114 (rabbit).
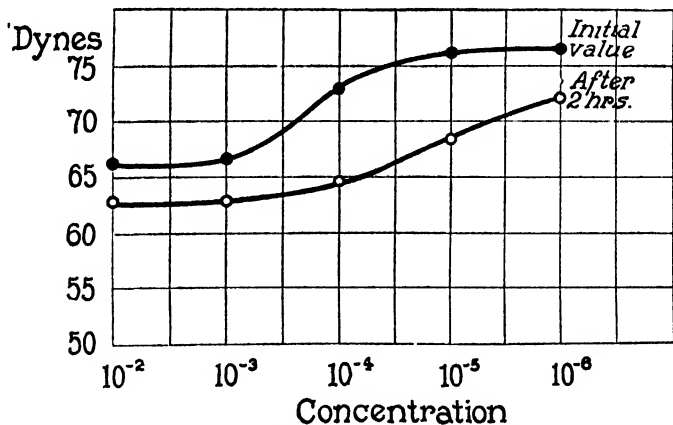
TEXT-FIG. 5. Values of the surface tension of serum solutions in function of the time. Serum 117 (rabbit).

The decrease of surface tension of solutions of organic compounds, such as glycocholate for example, follows the same law as that of serum
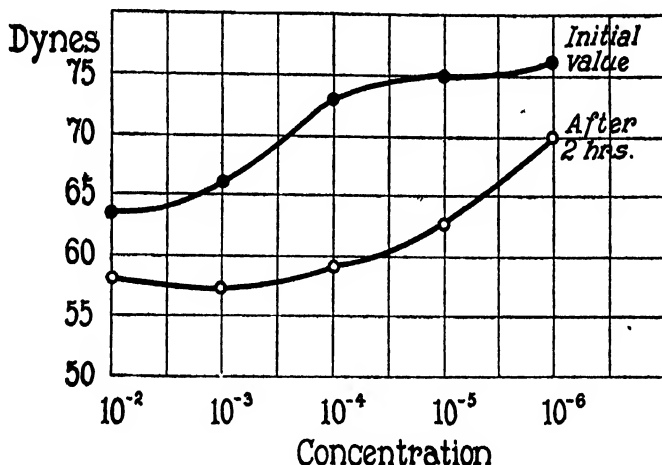
TEXT-FIG. 6. Values of the surface tension of serum solutions in function of the time. Serum 1 (dog).
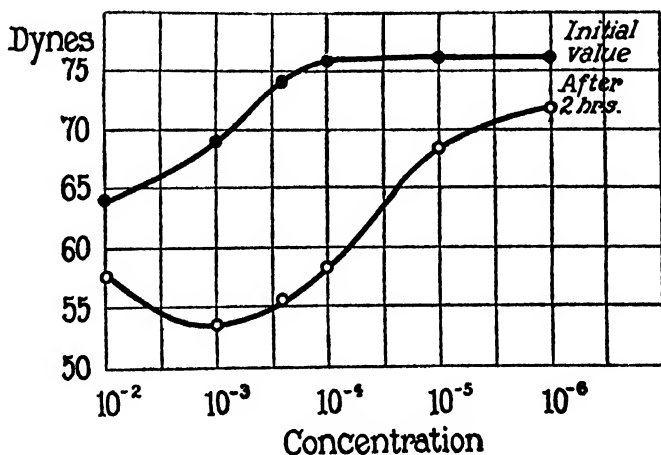
TEXT-FIG. 7. Values of the surface tension of serum solutions in function of the time. Young dog serum.

and is also very rapid at the beginning (Text-fig. 20). In this case the glycocholate was dissolved in distilled water (concentration 10^{-4}). When serum is diluted in distilled water, the same phenomenon occurs

(Text-figs. 21 and 22).   The maxima and the comparison with serum
are shown in Text-figs. 23 and 24.
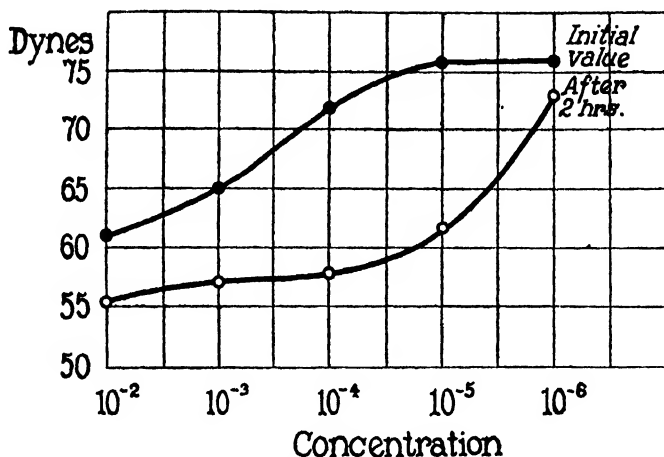
TEXT-FIG. 8. Values of the surface tension of serum solutions in function of the
time.   Human serum.
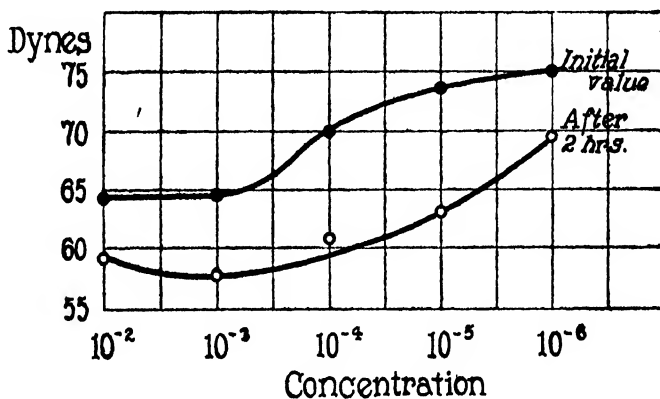
TEXT-FIG. 9. Values of the surface tension of serum solutions in function of the
time.   Serum 118 (rabbit).

It may be of interest to refer the reader to the work of Berczeller[3] on the surface
tension of colloidal solutions and, in general, to the numerous papers published by
Traube on the same subject.   The book by Freundlich,[4] a great number of papers

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[3] Berczeller, L., *Biochem. Z.*, 1913, liii, 215; 1914, lxvi, 173; *Internat. Z. physik.-
chem. Biol.*, 1914, i, 124.

[4] Freundlich, H., Kapillarchemie: Eine Darstellung der Chemie der Kolloide und
verwandter Gebiete, Leipsic, 1909.
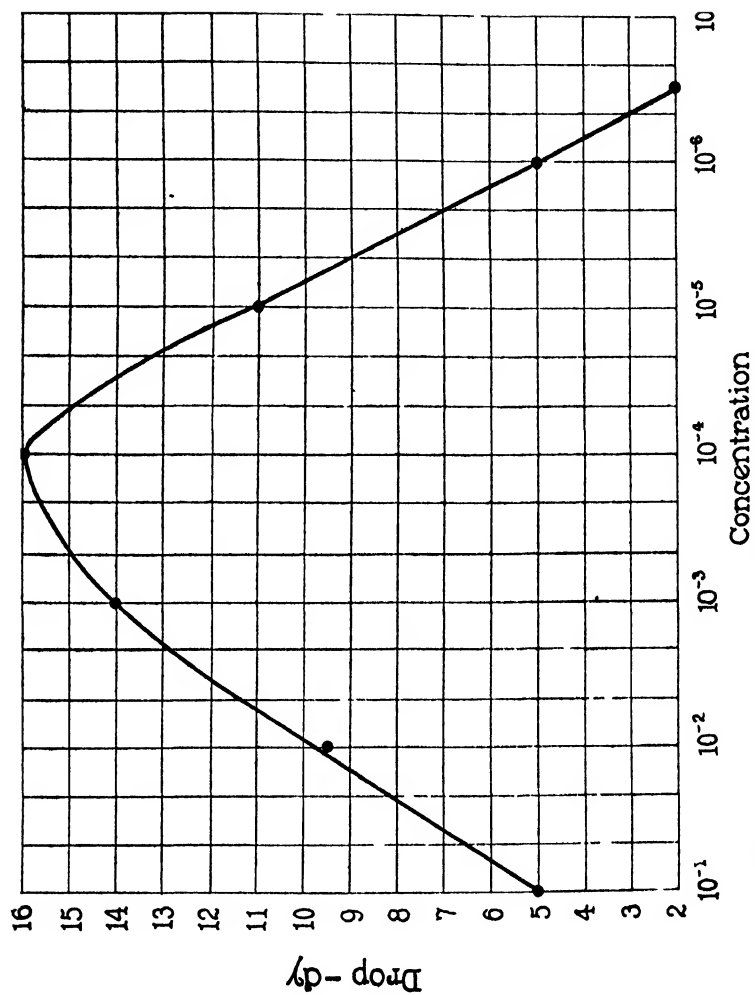
Text-Fig. 10. Drop in the surface tension of serum solutions in 2 hours. Serum 114 (rabbit).
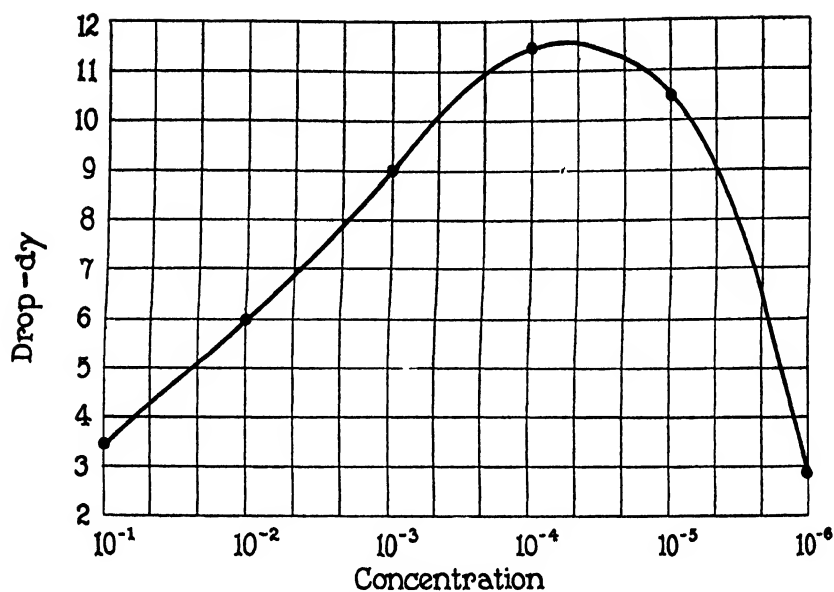
Text-Fig. 11. Drop in the surface tension of serum solutions in 2 hours. Serum 116 (rabbit).
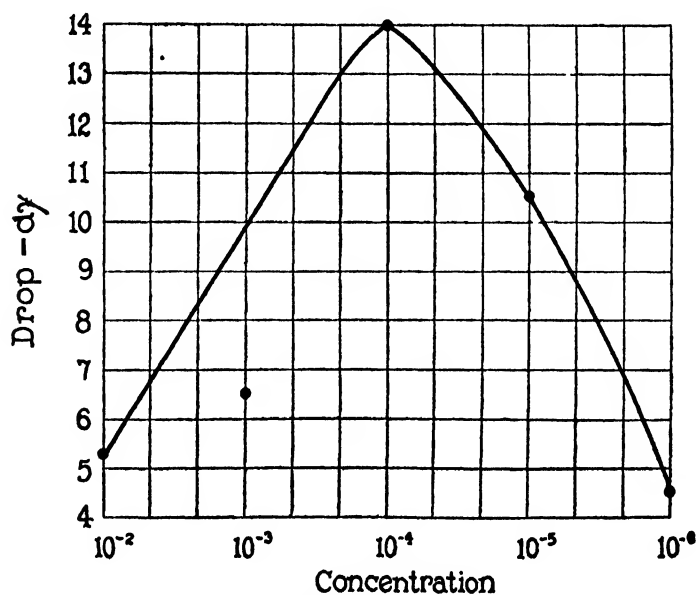
Text-Fig. 12. Drop in the surface tension of serum solutions in 2 hours. Serum 118 (rabbit).
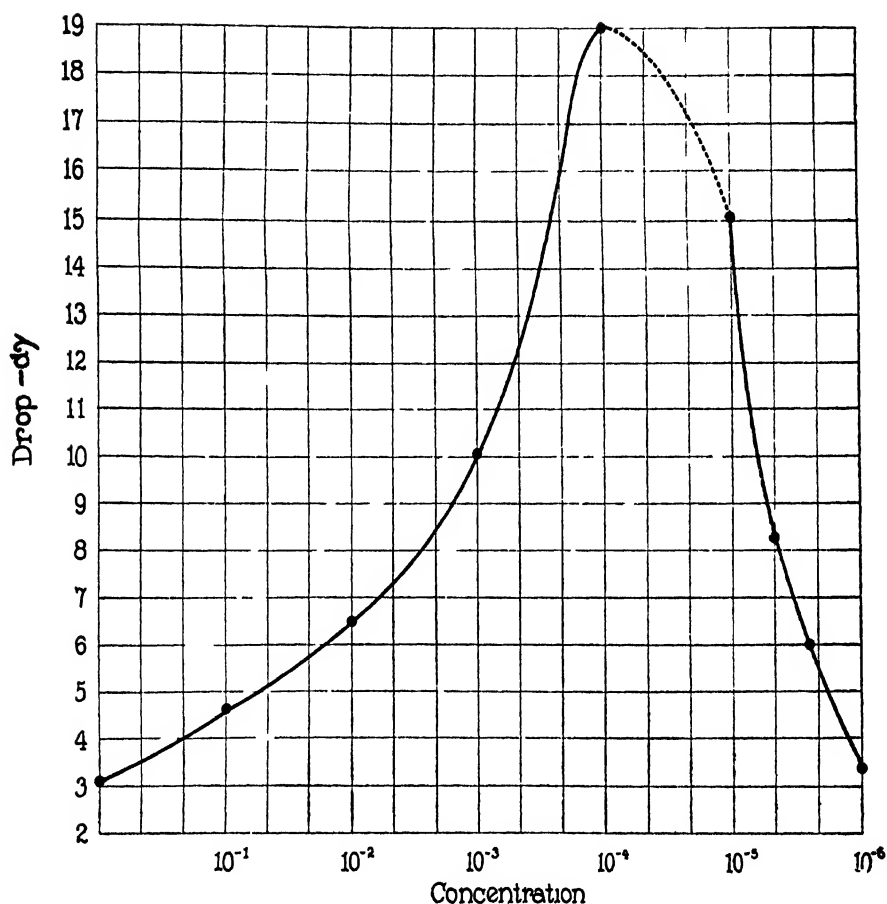
TEXT-FIG. 13. Drop in the surface tension of serum solutions in 2 hours. Old dog serum. As no measurements were made at concentrations between 10^{-4} and 10^{-5} a dotted line is used to connect these two points, since it is probable that the maximum would have been somewhere between these two concentrations. Therefore it is likely that the dotted line does not represent the exact curve.

in the *Internationale Zeitschrift für physikalisch-chemische Biologie*, and the papers by Posternak[5] are also worth perusal.   It seems that the solution of the problem has been postponed mainly by the confusion of the action of time with that of heat and of chemical reactions.   Therefore, it is important to determine, in all studies on colloidal solutions, the part due to their colloidal state and that which may be due to really specific actions (for instance the inactivation of serum by heating at 56° C.).
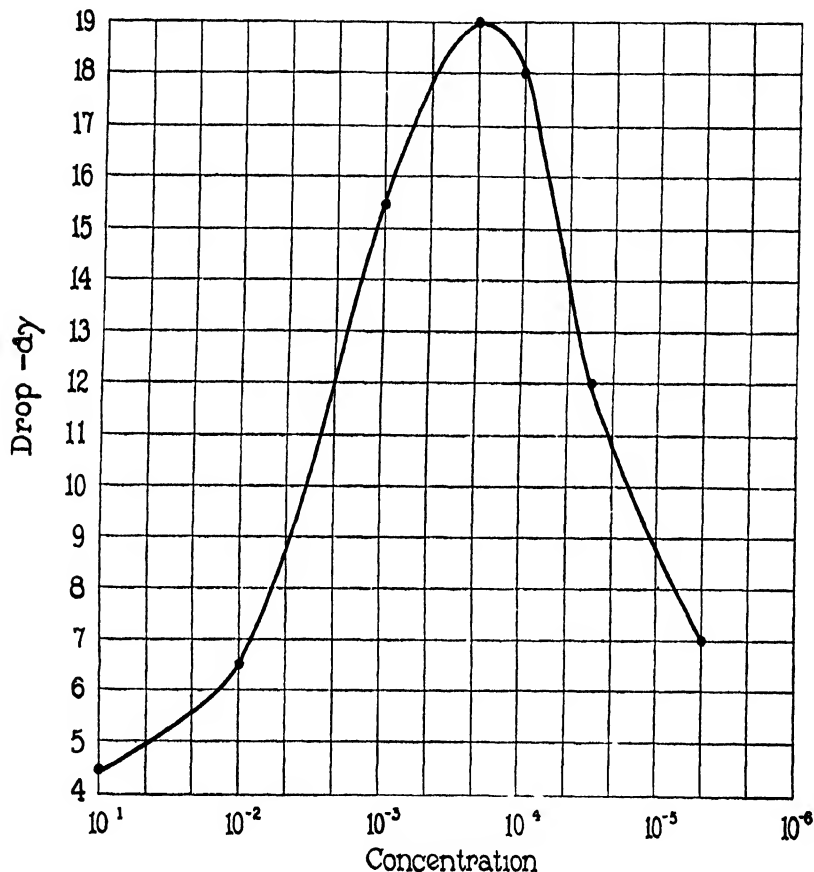
TEXT-FIG. 14. Drop in the surface tension of serum solutions in 2 hours. Young dog serum.

Thus, by merely introducing the factor time, are explained some of the discrepancies hitherto unaccounted for between the values of surface tension of solutions of organic compounds, as given by static

[5] Posternak, S., *Ann. Inst. Pasteur*, 1901, xv, 85, 169, 451, 570.

and dynamic methods. Indeed, certain methods, such as the capillary ascension for instance, measure the surface tension of the same layer of liquid in which adsorption in function of the time takes place, while
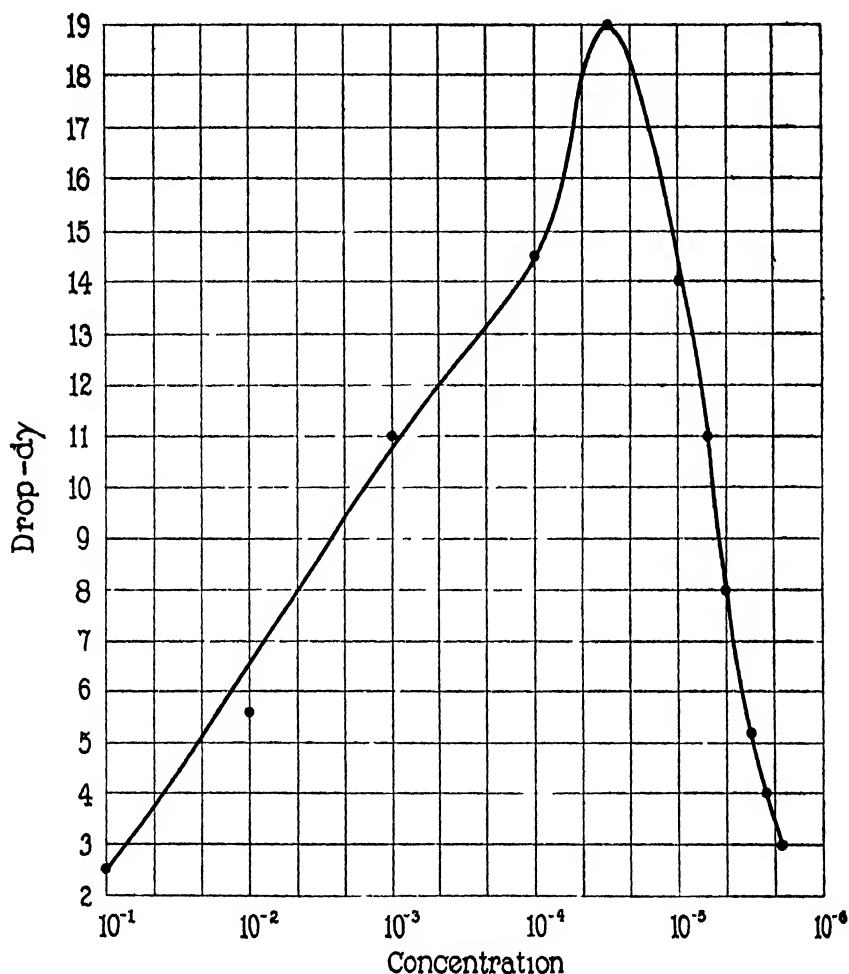
TEXT-FIG. 15. Drop in the surface tension of serum solutions in 2 hours. Human serum.

most dynamic methods, such as the undulatory jet methods, deal with a continuously renewed surface. The drop weight method' is intermediate, the drops requiring a longer time to fall. It is quite obvious that none of these last methods could show the drop in
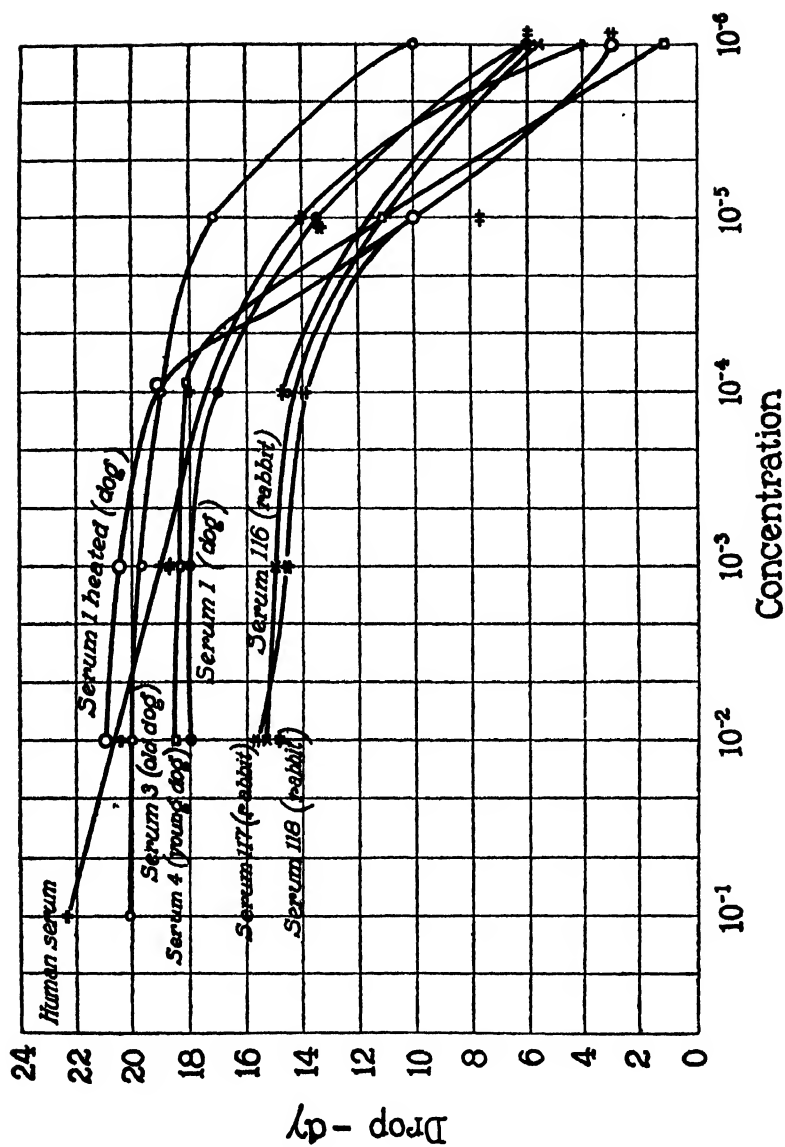
TEXT-FIG. 16. Drop in the surface tension of solutions of different sera in 2 hours. Value after 2 hours subtracted from 76.0 dynes.

TABLE IV.

*Decrease of Surface Tension of Solutions of Sodium Oleate, Sodium Glycocholate,
and Saponin in a 0.9 Per Cent Saline Solution in Function of Time.*

Sodium Oleate (Text -Fig. 17).

Dilution............................	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension.........	33.2	39.5	54.2	59.1	75.1
After 2 hrs..................	31.5	34.5	35.5	39.0	58.6

Sodium Glycocholate (Text-Fig. 18).

Dilution............................	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension.........	44.0	41.5	55.6	68.0	72.0
After 2 hrs..................	43.4	41.0	41.5	60.6	60.0

Saponin (Text-Fig. 19).

Dilution............................	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension.........	56.8	54.0	54.2	69.0	76.0
After 2 hrs..................	60.5	53.5	49.5	56.0	65.0
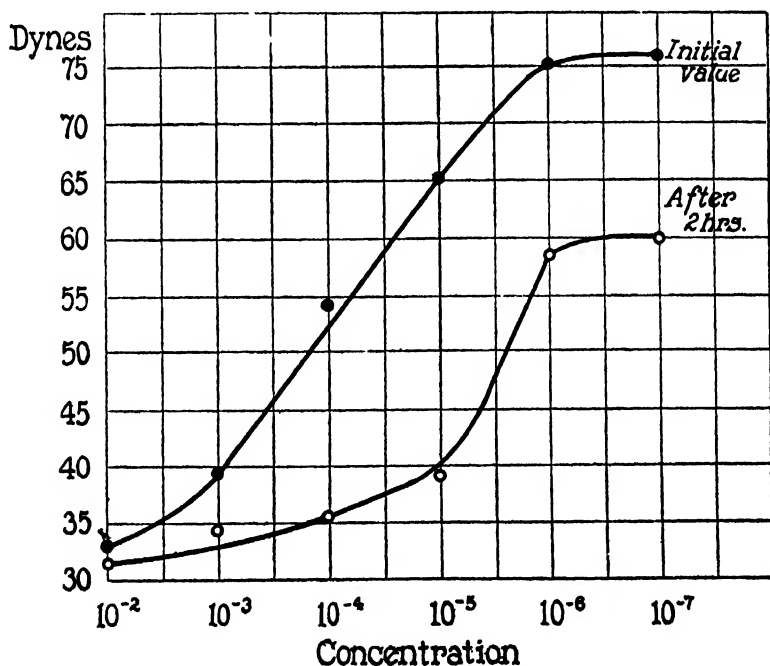
TEXT-FIG. 17. Values of the surface tension of solutions of sodium oleate in saline solution.
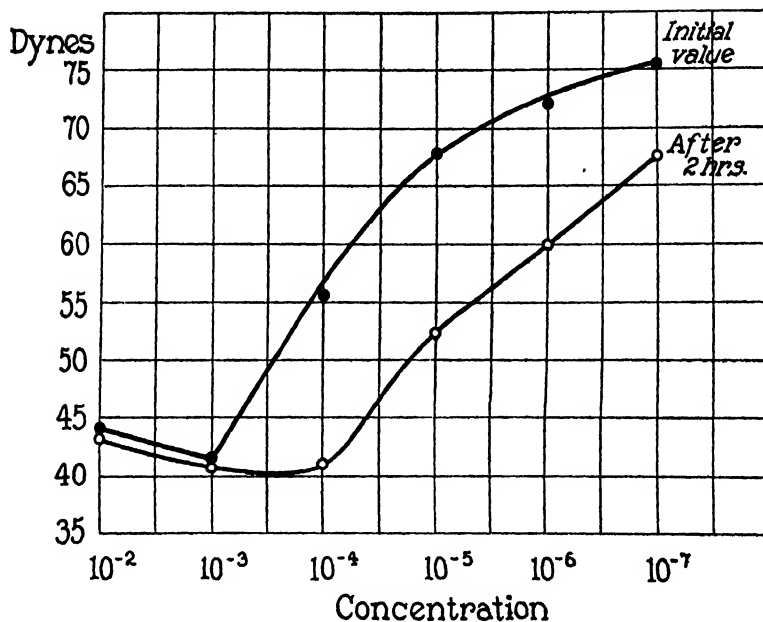
TEXT-FIG. 18. Values of the surface tension of solutions of sodium glyco-cholate in saline solution.
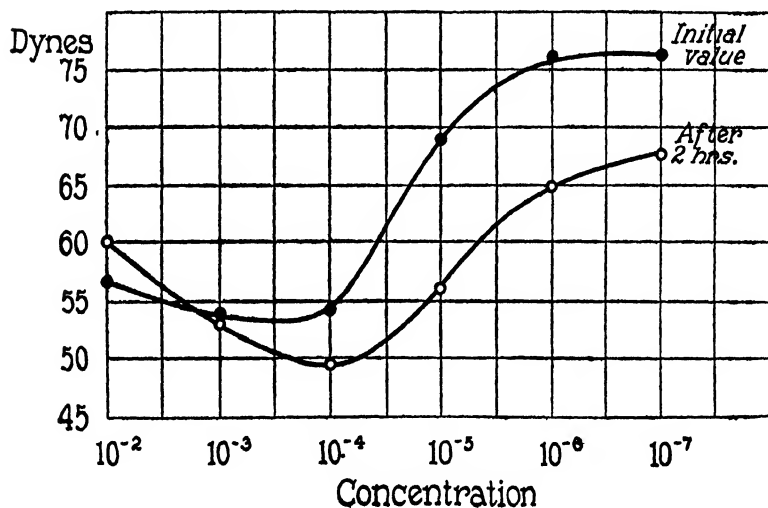
TEXT-FIG. 19. Values of the surface tension of solutions of saponin in saline solution.
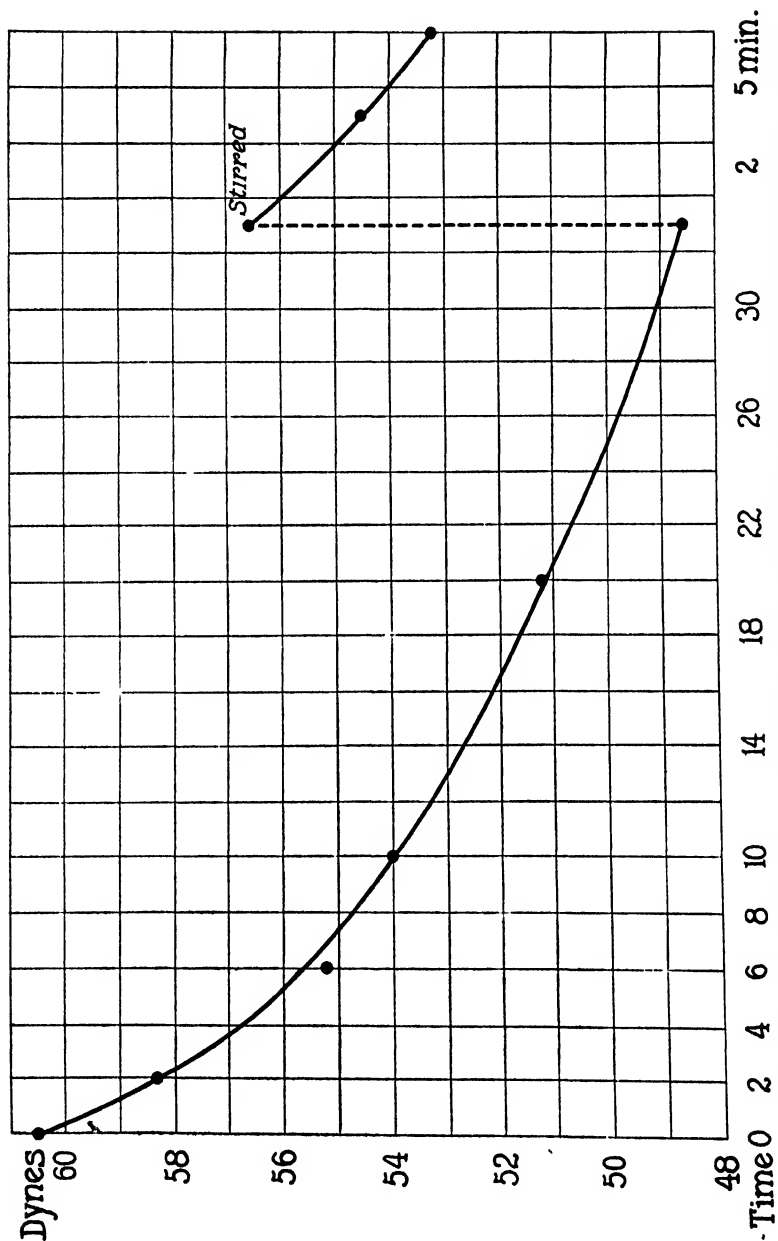
Text-Fig. 20. Values of the surface tension of solutions of sodium glycocholate in distilled water. Drop in the first 30 minutes; effect of stirring. Concentration 1:10,000.
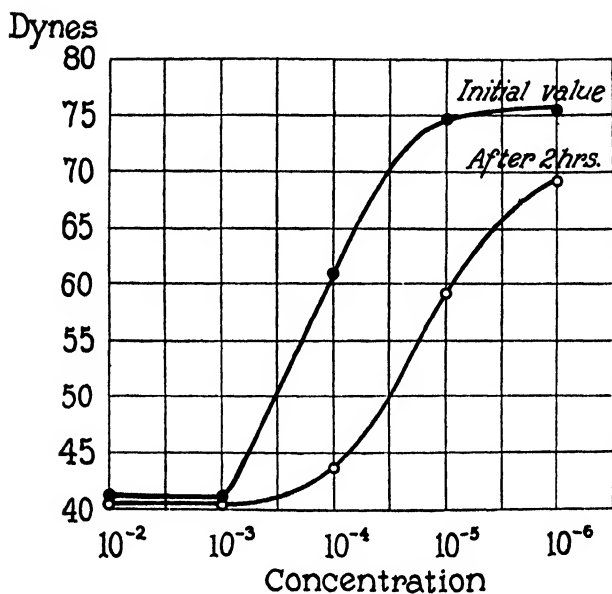
TEXT-FIG. 21. Values of the surface tension of solutions of sodium glycocholate in distilled water.
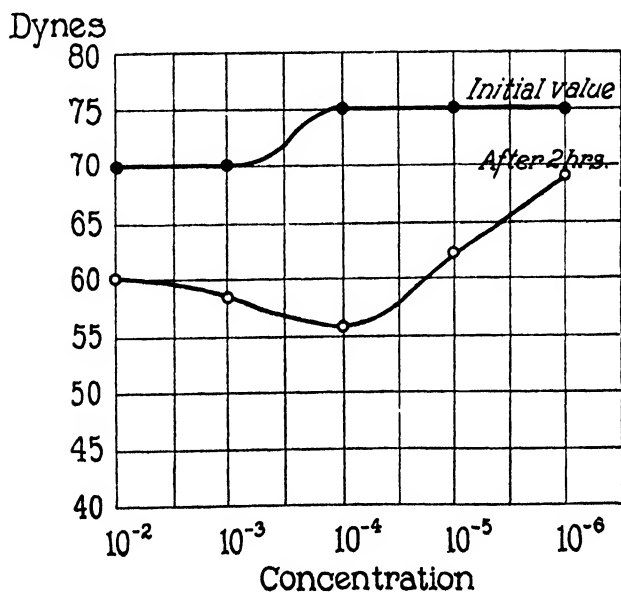
TEXT-FIG. 22. Values of the surface tension of solutions of serum in distilled water. Serum 118 (rabbit).

surface tension which occurs after a few minutes. With a solution of
1:100,000 the number of drops was the same as for pure saline solution:
17 drops whenever the measurement was made with a Traube stalag-
mometer (after a few seconds, 10 minutes, 2 hours, or 24 hours).
The tensiometer measured for the same intervals: 75.5, 66.0, 61.0,
55.5 dynes; that is, a drop of 20 dynes in 24 hours.
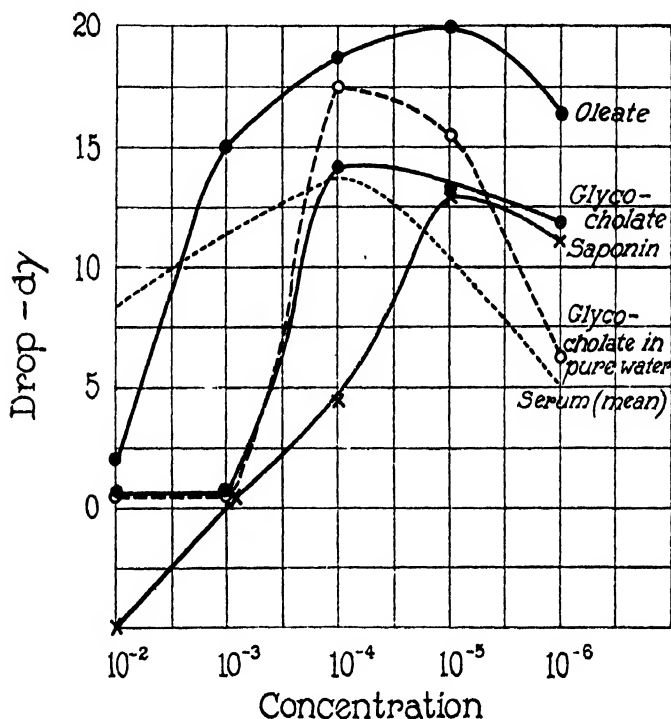
TEXT-FIG. 23. Drop in the surface tension of different solutions as expressed by
the formula

$$-d\gamma = \gamma_o - \gamma$$

3. *Effect of Stirring.*—When stirred, these solutions present the
same phenomenon as the pure serum; the surface tension rises and
sometimes reaches the same value as at the beginning of the experi-
ment (Table V and Text-figs. 25 and 26). When the solutions are
kept in test-tubes, that is with a relatively small free surface, vigorous
stirring after 24 hours brings the surface tension to the same value
(Table VI). This seems to indicate that the damping effect,
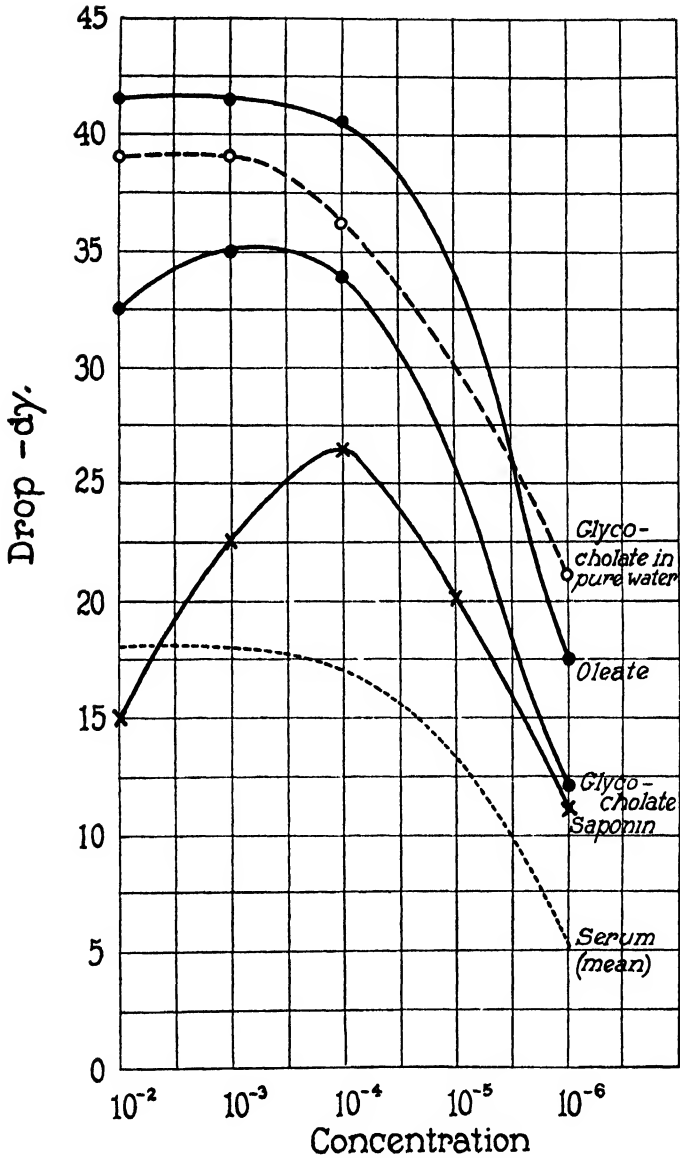
TEXT-FIG. 24. Drop in the surface tension of different solutions as expressed by the formula

$$-d\gamma = 76 - \gamma$$
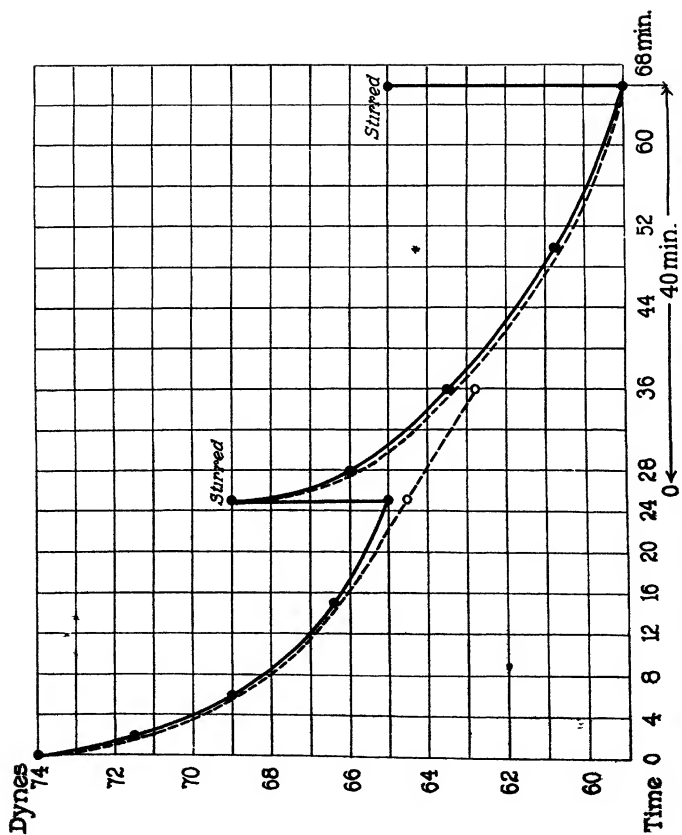
Text-Fig. 25. Effect of stirring on solutions of serum. Dilution 1:10,000. Serum 2 (dog). The dotted line is calculated according to the formula
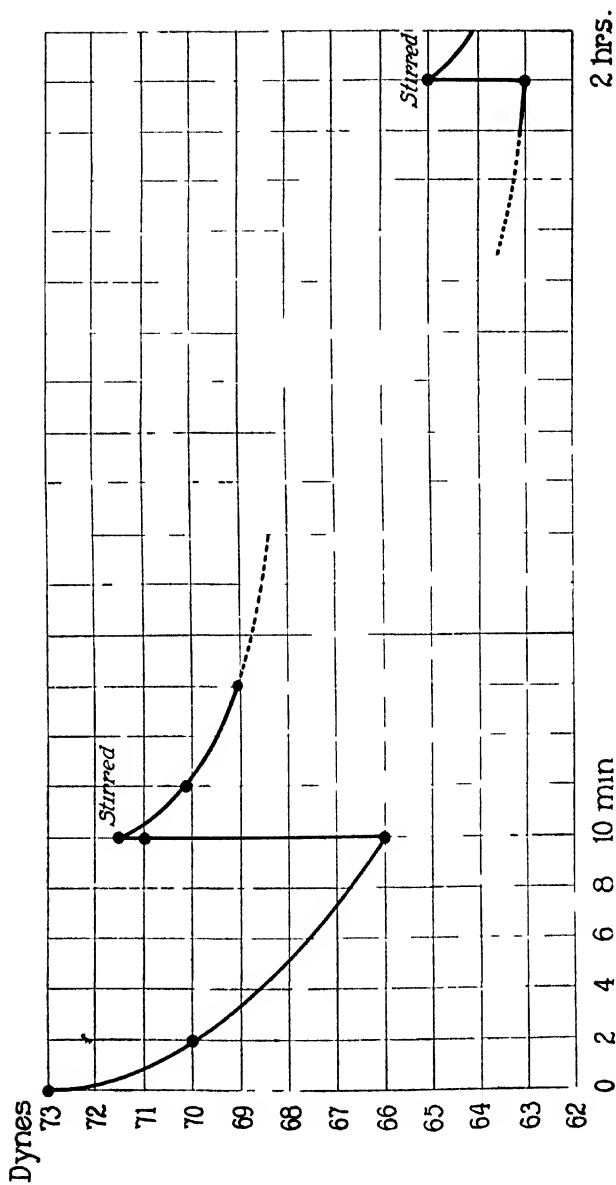
Text-Fig. 26. Effect of stirring on solutions of serum. Dilution 1:10,000. Serum 112 (rabbit). The interruption of the curve after the 22nd minute was necessary to bring the end of the curve after 2 hours within the same chart, without making it too long.

TABLE V.

Effect of Stirring on the Surface Tension of Serum Solutions.

Dog Serum, No. 2 (Text-Fig. 25).

Temperature 22° C.

Time	0	25 min.	Stirred.	40 min.	Stirred.
Dilution. 1:10,000	74.0	64.9	69.0	59.0	65.0

Rabbit Serum, No. 112 (Text-Fig. 26).

Temperature 22° C.

Time	0	10 min.	Stirred.	2 hrs.	Stirred.
Dilution. 1:10,000	73.0	66.0	71.5	63.0	65.0

Rabbit Serum, No. 118.

Temperature 23° C.

Time	0	24 hrs.	Stirred vigorously.
Dilution. 1:10,000	76.0	68.2	75.1

TABLE VI.

Effect of Stirring on the Surface Tension of Serum Solutions.

Dog Serum, No. 3, Kept in Test-Tubes.

Temperature 23° C.

Time	0	24 hrs., stirred vigorously.
Dilution.		
1:10	61.9	62.0
1:100	62.5	60.0
1:1,000	66.2	66.0
1:10,000	76.0	76.0
1:100,000	74.0	74.0

described in our previous paper,[6] is due to chemical changes under-gone in the surface layer. When the surface layer is small with respect to the bulk, the changes are undetectable.

III.

DISCUSSION.

1. Interpretation of the Maxima.—From Table I, as well as from Text-figs. 10 to 15, it is clear that the drop in the value of the surface tension is variable and reaches a maximum at a dilution of about 1:10,000. In general, this occurs only when the measured initial value of the tension of the serum is taken into consideration. In other words, if we assume the initial value of the tension to be around 76 dynes, as in the higher dilutions, there would not always be a·maximum. Therefore, we assume provisionally that the actually initial surface tension of such dilutions as 1:10, 1:100, 1:1,000, 1:10,000, is that which can be measured within 5 or 10 seconds after the liquid is poured into the watch-glass. It is very likely that there are at least two groups of substances which act powerfully upon the surface tension of the serum, and in different ways. The first group, in conformity with the substances already known, is adsorbed almost instantaneously in the surface layer, and determines the value of the surface tension of fresh serum and of serum solutions up to about 1:50,000, as given by the drop weight methods (with continu-ously renewed free surfaces); the other group which, on account of the techniques in use up to the present time, could not be detected is adsorbed in function of time, provided the free surface is left un-stirred. This second substance, or group of substances, acts more powerfully than the other, and at extremely low concentrations. Indeed, we have seen that one-millionth part of serum is still active. Since serum does not usually contain as much as 10 per cent of solid substances in solution altogether, we may assume, and this is mani-festly exaggerated, that the serum contains 5 per cent of these sub-stances. Hence, this would mean that they are still active at such a low concentration as 1:20,000,000 (1 gm. in 20,000 liters, or over 5,200 gallons of water). The hypothesis of the two groups of sub-

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[6] du Noüy,[1] p. 579.

stances is expressed by Text-figs. 10 to 15, in which the initial value of the surface tension has been taken into consideration for the evaluation of the drop. By assuming that it is the same substance, or group of substances, which determines the initial value of the surface tension by very rapid adsorption, and the slow drop by slower adsorption, we obtain Text-fig. 16.

Hence, admitting the correctness of the assumption of the existence of two groups of substances, it becomes easy to account for the special grouping of the initial values of surface tension. Indeed, if we call the first group A, and the second group B, it is readily seen, by looking at the charts, that Group A, present at dilutions from 1:10 to 1:1,000 in sufficient quantities to have a marked action on the tension, is no longer effective in the case of high dilutions, from 1:100,000 up. There is a break in the initial value which is not always bridged by the 1:10,000 dilution.   From 1:100,000 up, practically only Group B is active.   On the other hand, we notice that it is precisely around the concentration of 1:10,000 that the drop is maximum.   The reason is simple if we refer to Langmuir's ideas on the structure of the surface layer of solutions, when the solute is adsorbed in the surface layer. Langmuir[7] has shown, indeed, that the free surface of such a liquid is generally composed of a monomolecular layer in which the molecules *are geometrically disposed and similarly oriented with reference to a three dimensional space; in other words, that the surface of the liquid is composed of a sort of mosaic, each element of which is the same end of the same side of the molecules of the liquid.*   Furthermore, he states that the group molecules of organic liquids arrange themselves in such a way that their active portion is drawn inwards, leaving the least active portion of the molecule to form the surface layer. This hypothesis is supported by very striking facts, but their description would be beyond the scope of this paper.

Thus, in the case of serum solutions, the maximum drop would correspond to such a dilution as would allow the active molecules of Group B to dispose themselves with what is left of Group A in the most favorable way for the decrease of surface tension; and this is only possible at one precise concentration.   At higher concentrations, the

[7] Langmuir, I., *Met. Chem. Eng.*, 1916, xv, 468; *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

excess of molecules of Group A interferes with the best possible geometrical arrangement of the molecules of Group B, and the forcing apart of these groups increases the surface tension. The same thing happens at higher dilutions, in which the forcing apart is due to the smaller number of molecules of Group B, which can no longer cover the liquid completely. This is, of course, a mere hypothesis which has, at least, the advantage of accounting for the facts found so far. But, whatever hypothesis is accepted, and in spite of the fact that the maximum drop is not so clear if the presence of only one group of substances acting together in the same way is assumed, there is no doubt but that an optimum concentration exists; it is easily explained, as well as the grouping of the initial values, on the basis of Langmuir's conception of the structure of liquid layers.

A glance at the charts will also show that the maximum drop does not always occur at the same concentration. Assuming the correctness of our hypothesis, this shift may be due to a change in the size of the group molecules. Indeed, the maximum drop occurs when the free surface is entirely covered with a certain lattice of molecules of Groups A and B, arranged in such a way as to produce the weakest force fields. Evidently there is an optimum concentration, as has been said before. But this optimum is function of the size of the molecules, and of the relative concentration of the two groups, A and B. This will be more clearly understood by looking at Text-fig. 27, a to c. In Text-fig. 27, a, the optimum is attained for a certain size of the molecules, and a certain relative number of both groups. The quantity adsorbed in the surface layer is exactly that which corresponds to the minimum surface tension. In Text-fig. 27, b, the size of the molecules is the same and the quantity adsorbed is greater, the concentration being higher. The arrangement of the molecule is no longer perfect, the proportion of Groups A and B is changed, and the molecules may be forced to tilt on one side, or to overlap. The result is that the surface tension is increased. In Text-fig. 27, c, we have another optimum arrangement, with a smaller number of larger molecules; that is, at a lower concentration. Although this illustration is very crude, it helps to visualize a plausible process through which the maximum may be shifted. As the molecules in solution are probably very long, with lateral branches, instead of

discs or spheres, as we have symbolically represented them, these figures must not be taken as an image of reality but as a mere comparison.

In the curves published, the maximum for human serum lies at 1:50,000, for the old dog serum at 1:30,000, for rabbit serum around 1:10,000, and for a very young pup at 1:7,000.
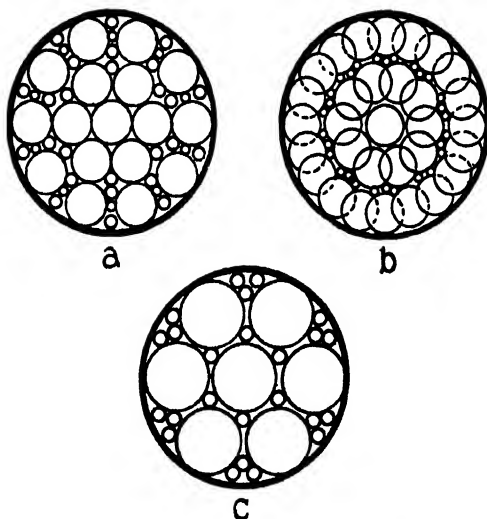
TEXT-FIG. 27, a to c. Hypothetical illustration of the possible arrangement of molecules in the surface layer of a serum solution.

IV.

Aspect of Crystallization of Serum Solutions.

When the solutions are allowed to evaporate, the sodium chloride crystallizes. By using watch-glasses, an interesting phenomenon is observed. When a pure sodium chloride solution crystallizes, it concentrates first, then the crystals are formed at the bottom of the glass as seen in Fig. 1. But when serum is added, very small crystals spread all over the watch-glass, and assume different aspects, according to the concentration, as shown by Figs. 2 and 3. Up to a concentration of 1:500,000 in the case of Serum 114, the effect of the lowering of the surface tension can be detected easily by the action of the substances adsorbed in the surface layer upon the molecules of sodium chloride. This phenomenon can be explained in the

following way. The large adsorbed molecules carry with them in the surface layer the Na and Cl ions. The concentration there becomes very high, and dissociation small. Sodium chloride molecules are formed and when the liquid evaporates, it progressively abandons the salt on the glass. No concentration occurs in the bulk, and no large crystals can grow at the bottom.

The phenomenon is similar when other surface-active substances are used, such as for instance saponin (Fig. 4).

This phenomenon is interesting because it shows that the presence of sodium glycocholate or taurocholate or dried serum in such a high dilution as 1:10,000,000 modifies profoundly the state of equilibrium and the distribution of phases in a liquid containing crystalloids in solution. Gibbs' statement that substances which increase the surface tension are more concentrated in the bulk is no longer true in this case. The birth of the membranes at interfaces, the first manifestation of the individuality of a cell, becomes very clear. The concentration of colloids and salts being much greater in the surface layer, precipitation will naturally occur there, and electric phenomena are forcibly different from those in the bulk. The aspect of the crystals is not the same at all dilutions, and at a concentration of 1:100, all sera studied so far have shown a very peculiar phenomenon, easily seen in the photographs. The more or less regular rings cannot be found as clearly at any other concentration. The white rings are very small sodium chloride crystals. The theoretical explanation of this phenomenon is not obvious. There is no doubt that it corresponds to different states of equilibrium of the surface layer, and possibly to different and periodical changes in its surface tension. This phenomenon is somewhat similar to that observed by Liesegang,[8] obtained by adding 1 drop of a silver nitrate solution to a solution of gelatin and potassium dichromate.

As was to be expected, sodium oleate, glycocholate, and saponin act in the same way. Further investigations are being conducted on this subject.

[8] Liesegang, R. E., Z. Chem. u. Indust. Kolloide, 1907–08, ii, 70.

V.

CONCLUSIONS.

The application of the ring method to the measurement of solutions of serum and of certain organic compounds has brought forth new facts, mainly the decrease of the surface tension of such solutions in function of time.

1. In serum diluted at such a low concentration as 1:1,000,000 in NaCl, physiological solution, the surface tension of the liquid is lowered by 3 or 4 dynes in 2 hours; at 1:100,000, by about 11 dynes (mean value) in 2 hours, and by 20 dynes in 24 hours; at 1:10,000 by about 13 to 16 dynes in 2 hours.

2. The drop in surface tension is much more rapid in the first 30 minutes and follows generally the law of adsorption in the surface layer in function of the time.

3. Stirring or shaking after the drop causes the surface tension to rise, but generally below its initial value.

4. The same phenomena occur when using sodium oleate, glycocholate, or saponin instead of serum.

5. For every serum, as well as for the substances mentioned above a maximum drop occurs in certain conditions at a given optimum concentration.

6. Not only are the substances which lower the surface tension adsorbed in the surface layer, in the case in which they are present with crystalloids, but also the crystalloids themselves, in contradiction to Gibbs' statement. This is plainly shown by the evaporation of such solutions in watch-glasses which, instead of a small group of sharp, large, well defined crystals at the bottom, leaves a white disc almost as large as the initial free surface itself, due to the liberation of the salt by the surface layer as it crawls down the concave surface of the glass.

7. In these conditions, solutions of serum are characterized by a very peculiar periodic and concentric distribution of the crystals, at a concentration of 1:100 only. The same ring-like aspect is observed with sodium oleate, glycocholate, and saponin, but not at the same concentration, as was to be expected, since serum is a solution in itself.

EXPLANATION OF PLATES.

PLATE 58.

FIG. 1. Crystals of serum solutions in NaCl solution at 0.9 per cent. Serum 114 (rabbit).

PLATE 59.

FIG. 2. Crystals of serum solutions in NaCl solution at 0.9 per cent. Serum 117 (rabbit).

PLATE 60.

FIG. 3. Crystals of serum solutions in NaCl solution at 0.9 per cent. Human serum.

PLATE 61.

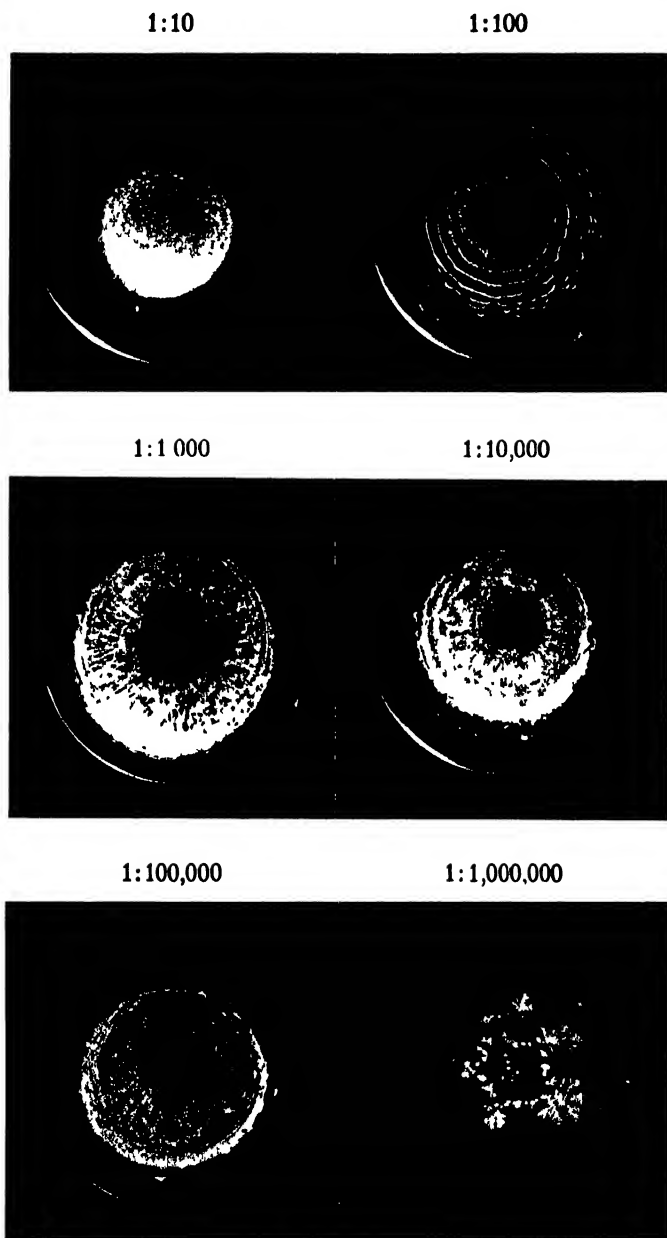FIG. 4. Crystals of saponin solutions in NaCl solution at 0.9 per cent.

1:100　　　　　　　1:1,000　　　　　　1:10,000

1 100,000　　　　　1 200,000　　　　　1:400,000

1 500,000　　　　　1 600,000　　　　　1 800,000

NaCl 9 per cent　　　　NaCl　　　　　　NaCl

Fig. 1.

1:10   1:100

1:1 000   1:10,000
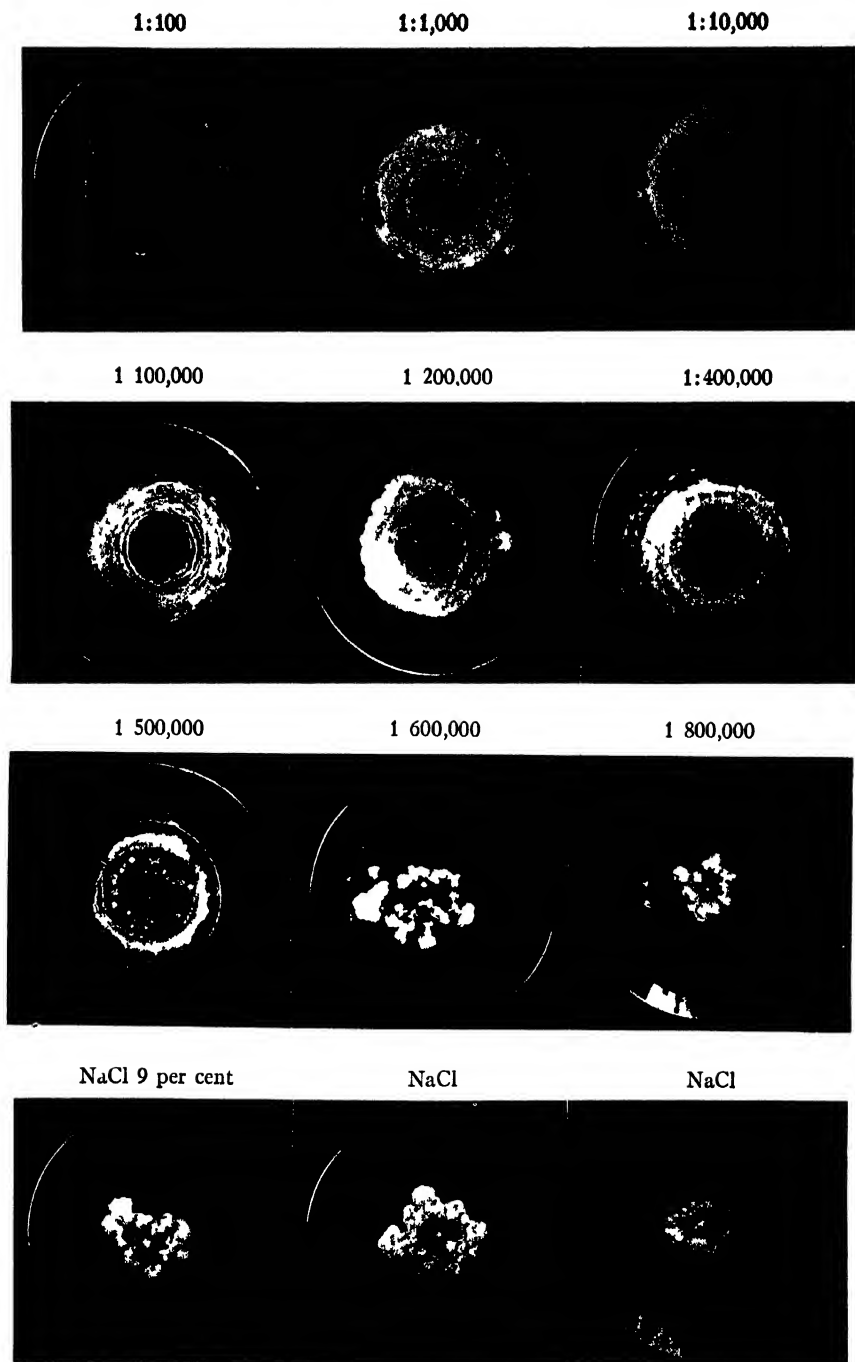
1:100,000   1:1,000,000

FIG. 2.

(du Noüy. Surface tension of serum. II.)
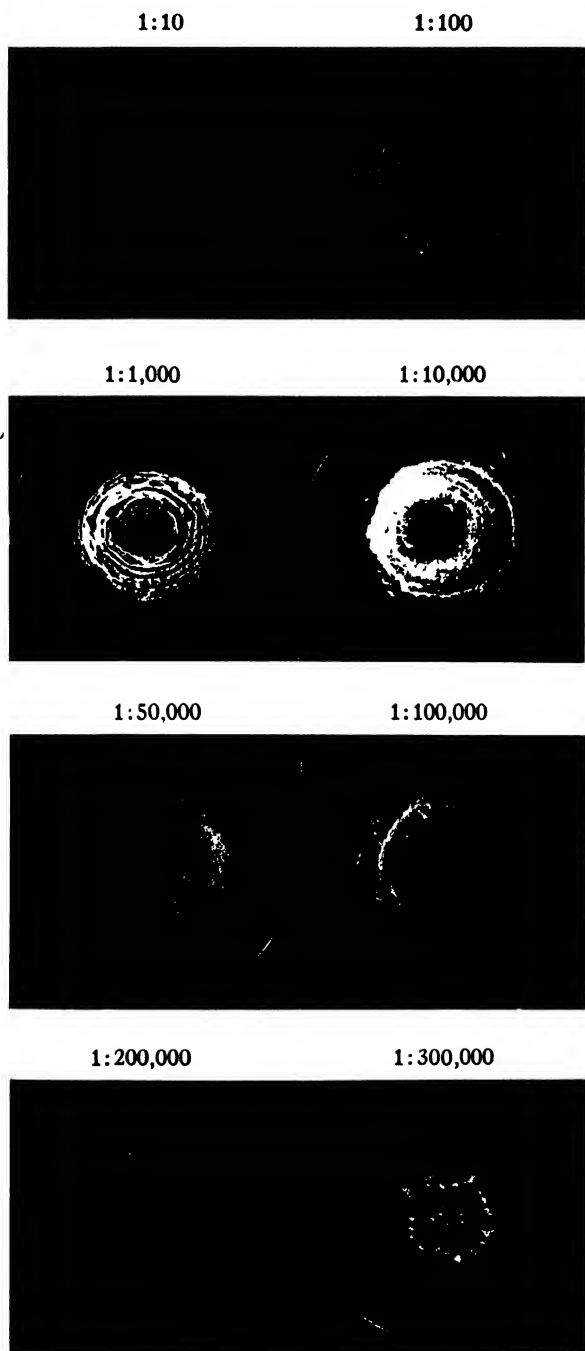
FIG. 3.

(du Noüy: Surface tension of serum.　II.)

1:100   1:1,000

1:10.000   1:100,000

1:1,000,000   1.10,000,000
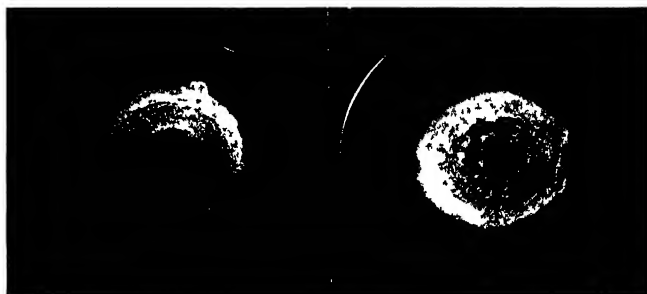
NaCl

Fig. 4.

HEAT AND GROWTH-INHIBITING ACTION OF SERUM.

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INTRODUCTION.

It is known that plasma or serum obtained from an adult animal restrains the growth of a pure culture of homologous fibroblasts.[1] This inhibiting power of plasma or serum is not apparent in very young animals, but manifests itself a few weeks after birth, increases progressively during adult life, and becomes very marked in old age. If it should restrain cell proliferation *in vivo* as much as *in vitro*, its rôle in many physiological and morbid processes would be important. Therefore, an investigation of its nature was begun. The purpose of the experiments described in this article was to study the modifications occurring in the rate of growth of fibroblasts when the serum composing the culture medium had been heated at various temperatures.

EXPERIMENTAL.

The serum was obtained from the plasma of chickens about 1 year old which had fasted for 24 hours. Its inhibiting action was determined by comparing the rate of growth of fibroblasts in a medium containing 50 per cent serum and in another containing 50 per cent Tyrode solution. The growth index of this serum averaged 0.9, which indicated that the inhibiting power was slight. The serum was used in the preparation of the medium in two different concentrations. When the fibrin of the coagulum was obtained from plasma, the medium was composed of 33.3 per cent plasma, 4 per cent tissue juice, and 62.6 per cent serum. When fibrinogen was used instead of plasma, the medium was made of 20 per cent fibrinogen suspension,[2]

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[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

[2] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

50 per cent serum, 27.5 per cent Tyrode solution, and 2.5 per cent tissue juice.

The action of unheated serum was compared with that of heated serum. Serum heated at 56°C. for a period of time varying from $\frac{1}{2}$ to 24 hours became slightly opalescent. When it was heated at 70°C. for $\frac{1}{2}$ hour, a precipitate occurred which was thrown down by centrifugation. The supernatant fluid was transparent, slightly opalescent, and not very viscous. The serum heated at 100°C. coagulated. The fluid which remained around the coagulum was used in the preparation of the medium.

The fibroblasts were taken from stock cultures of a 9 year old strain of connective tissue, and the cultures prepared in the usual manner. They were allowed to grow for 48 hours and the measurements were taken by outlining the original fragment and the new tissue under the projection apparatus and determining the area with the planimeter. The relative increase was calculated.[3]

1. Action of Serum Heated at 56°C.—In the first series of experiments, a comparison of the action of sera unheated and heated at 56°C. for 1 hour was made in media containing 33.3 per cent chicken plasma (Tables I and II). The rate of growth of fibroblasts in both media was identical.

In a second series of experiments, 20 per cent fibrinogen suspension was used instead of plasma. Differences in the rate of growth under the influence of heated and unheated sera became apparent (Tables III to VIII). The time during which the serum was kept at the temperature of 56°C. had little influence on its inhibiting action. The rate of growth of fibroblasts in sera heated at 56°C. for $\frac{1}{2}$ to 24 hours was decreased about 15 per cent.

2. Action of Serum Heated at 70°C.—In the first series of experiments, the action of serum heated at 70°C. for $\frac{1}{2}$ hour was compared with that of serum heated at 56°C. (Table IX). The rate of growth in the serum heated at 70°C. was 22 per cent slower than in the serum heated at 56°C.

In a second series of experiments, the action of sera unheated and heated at 70°C. was compared (Table X). The growth was found to

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

TABLE I.

Rate of Growth of Fibroblasts in Plasma and Serum, Unheated and Heated at 56°C.

Experiment No.	Culture No.	Width of ring.	
		Serum 62.6 per cent.	
		Unheated.	Heated.
1	23191	5.2	4.9
2	23192	4.9	5.1
3	23193	4.6	4.7
Average		4.9	4.9
4	1185–1, 1185–2	5.0	4.5
5	1185–3, 1185–4	4.0	4.0
Average		4.5	4.25
6	23194	5.0	4.5
7	23195	4.0	4.5
8	23196	5.0	5.0
Average		4.7	4.7

The serum used in the first three experiments was heated at 56°C. for 1 hour, in the remaining five experiments, for 2 hours. The rate of growth is expressed by the width of the ring of new tissue.

TABLE II.

Rate of Growth of Fibroblasts in Plasma and Serum. Unheated and Heated at 56°C.

Experiment No.	Culture No.	Relative increase.		Ratio: $\dfrac{\text{Heated serum}}{\text{Unheated serum}}$.
		Serum 62.6 per cent.		
		Unheated.	Heated.	
1	23191	4.08	4.02	0.99
2	23192	3.76	3.91	1.04
3	23193	3.46	4.05	1.17
Average				1.07
4	23194	3.85	3.61	0.94
5.	23196	3.82	4.07	1.07
Average				1.00

In the first group of experiments, the serum was heated at 56°C. for 1 hour, in the second group, for 2 hours. The rate of growth is expressed by the relative increase of the tissue.

TABLE III.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for ½ Hr.

Experiment No.	Culture No.	Relative increase. Serum 50 per cent. Unheated.	Heated.	Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$.
1	20936	3.36	2.72	0.80
2	20937	2.13	1.62	0.76
3	20938	3.50	2.68	0.77
4	21491	5.06	4.87	0.96
5	21492	3.43	2.93	0.85
6	21493	4.80	3.43	0.71
7	21450	2.76	2.51	0.91
8	21841	2.90	2.20	0.76
9	21842	2.60	2.40	0.92
10	21843	2.80	2.30	0.82
11	21844	2.40	2.40	1.00
12	23110	2.16	1.89	0.88
Average..................		3.16	2.66	0.85

TABLE IV.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 1 Hr.

Experiment No.	Culture No.	Relative increase. Serum 50 per cent. Unheated.	Heated.	Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$.
1	23164	3.72	3.42	0.92
2	23165	3.73	3.37	0.90
3	23166	2.91	2.91	1.00
4	23167	2.48	2.30	0.93
5	23226	3.55	4.00	1.12
6	23227	4.35	4.47	1.03
7	23246	2.28	1.81	0.79
8	23248	2.74	2.00	0.73
9	23249	2.21	1.85	0.84
Average.............................				0.92
10	23477	5.12	4.64	0.91
11	23478	3.92	3.55	0.91
12	23479	3.80	3.12	0.82
13	23944	2.80	2.22	0.79
14	23945	3.21	2.65	0.83
15	23946	3.14	2.56	0.82
Average..................................				0.85

In the first nine experiments, 5 per cent embryo juice was used, and in the remaining six experiments, 2.5 per cent embryo juice.

TABLE V.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 2 Hrs.

Experiment No.	Culture No.	Relative increase. Serum 50 per cent.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Unheated.	Heated.	
1	23168	3.12	2.77	0.89
2	23169	3.00	2.74	0.91
3	23170	3.19	2.67	0.84
4	23171	3.10	2.83	0.91
5	23228	3.50	3.22	0.92
6	23229	3.93	3.80	0.97
7	23251	3.24	2.85	0.88
8	23252	2.55	2.33	0.91
9	23253	3.32	3.00	0.90
Average.........				0.90

In the first two experiments, 5 per cent embryo juice was used, and in the remaining seven experiments, 2.5 per cent embryo juice.

TABLE VI.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 6 Hrs.

Experiment No.	Culture No.	Relative increase. Serum 50 per cent.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Unheated.	Heated.	
1	23288	3.21	2.70	0.84
2	23289	4.21	3.20	0.76
3	23290	4.05	3.21	0.79
Average				0.80

TABLE VII.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 12 Hrs.

Experiment No.	Culture No.	Relative increase. Serum 50 per cent.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Unheated.	Heated.	
1	23291	3.50	2.78	0.79
2	23292	3.70	3.11	0.84
3	23293	3.67	3.22	0.88
Average				0.84

TABLE VIII.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 24 Hrs.

Experiment No.	Culture No.	Relative increase.		Ratio: $\dfrac{\text{Heated serum}}{\text{Unheated serum}}$.
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23440	3.70	3.08	0.83
2	23441	3.58	2.93	0.82
3 .	23442	3.87	3.03	0.78
4	23334	3.07	2.90	0.94
5	23335	3.26	3.15	0.96
6	23336	3.48	2.90	0.83
Average...				0.86

TABLE IX.

Rate of Growth of Fibroblasts in Serum Heated at 56°C. for ½ Hr. and at 70°C. for ½ Hr.

Experiment No.	Culture No.	Relative increase.		Ratio: $\dfrac{\text{Serum heated at 70°C.}}{\text{Serum heated at 56°C.}}$.
		Serum 50 per cent.		
		Heated at 56°C.	Heated at 70°C.	
1	23812	2.70	1.98	0.73
2	23813	2.33	2.00	0.86
3	23814	3.06	2.56	0.84
4	23846	2.70	2.18	0.81
5	23847	2.56	1.92	0.75
6	23848	2.76	2.08	0.75
7 .	23886	3.26	2.76	0.85
8	23887	2.67	2.37	0.89
9	23888	3.00	2.20	0.73
10	23941	3.40	2.44	0.72
11	23942	3.40	2.43	0.71
12	23943	3.78	2.80	0.74
13	23947	2.39	1.74	0.73
14	23948	2.33	1.81	0.78
15	23949	2.84	2.28	0.80
Average...				0.78

TABLE X.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 70°C. for ½ Hr.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23337	3.20	2.28	0.71
2	23338	4.33	3.14	0.73
3	23339	4.24	2.69	0.63
4	23443	3.91	2.08	0.53
5	23444	3.43	1.69	0.49
6	23445	3.95	1.69	0.43
7	23709	3.62	2.25	0.62
8	23710	3.90	2.57	0.66
9	23711	3.90	2.60	0.67
10	23679	2.38	1.94	0.82
11	23680	2.21	1.80	0.81
12	23681	3.88	2.93	0.76
Average				0.66

be 34 per cent slower in heated than in unheated serum. Both series of experiments showed almost identical results. It must be observed also (Tables IX and X) that in both series the values for the relative increase of the tissues in serum heated at 70°C. are about similar. The consistency of the results demonstrates that there were no technical errors.

TABLE XI.

Rate of Growth of Fibroblasts in Serum Heated at 100°C. for 10 Min. and at 70°C. for ½ Hr.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated at 70°C.}}{\text{Heated at 100°C.}}$
		Serum 50 per cent.		
		Heated at 100°C.	Heated at 70°C.	
1	23889	3.35	2.30	0.69
2	23890	2.60	1.90	0.73
3	23891	3.26	2.55	0.78
Average				0.73

TABLE XII.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 100°C. for 10 Min.

Experiment No.	Culture No.	Relative increase. Serum 50 per cent.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$.
		Unheated.	Heated.	
1	23632	3.12	3.06	0.98
2	23633	3.43	3.01	0.88
3	23634	3.19	3.02	0.95
4	23635	2.88	2.85	0.99
5	23654	2.91	2.87	0.99
6	23655	3.05	3.44	1.13
7	23656	3.32	3.14	0.94
8	23657	3.30	2.90	0.88
9	23849	4.03	3.25	0.81
10	23850	3.16	2.74	0.87
11	23851	3.81	3.56	0.93
Average..				0.94
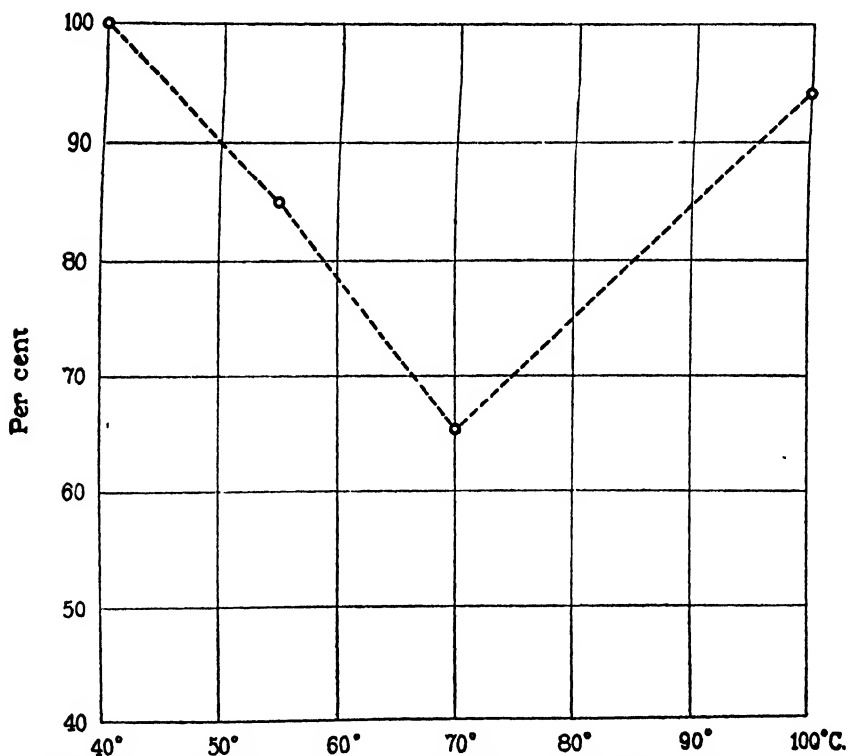
TEXT-FIG. 1. Variations in the rate of growth of fibroblasts in function of the temperature at which the serum was heated. The rate of growth of fibroblasts in unheated serum was considered to be equal to 100 per cent.

3. *Action of Serum Heated at 100°C.*—The fluid remaining after coagulation was compared with serum heated at 70°C. and with unheated serum. The figures show that the rate of growth in serum heated at 100°C. was 27 per cent faster than in serum heated at 70°C.; that is, about equal to the growth rate in unheated serum (Table XI). The experiments in which a comparison was made between the respective actions of unheated serum and serum heated at 100°C. confirmed these results (Table XII). The amount of growth was practically identical in both unheated serum and serum heated at 100°C. It must be remembered that differences equal to or smaller than 10 per cent may be due to experimental errors.

A curve was plotted expressing the variations of the rate of growth of the fibroblasts in function of the temperature at which the serum had been heated (Text-fig. 1). It shows that the rate of cell proliferation decreased after the serum had been heated at 56°C., and became still slower after it had been heated at 70°C. A marked increase in the rate of growth took place after the serum had been heated at 100°C.

DISCUSSION.

The results obtained in the preceding experiments may be summarized as follows:

The action of heat at 56°C. increased by 15 per cent the inhibiting action of serum obtained from young adult chickens on the proliferation of fibroblasts. The action of heat at 70°C. increased the inhibiting action by 34 per cent. When the serum had been heated at 100°C., its inhibiting action became about equal to that of non-heated serum. Therefore, heated serum contained a factor which markedly inhibited the growth of fibroblasts and which developed at, or resisted a temperature of 70°C.

These experiments confirmed the results obtained by Ingebrigtsen[4] in his study of the growth of guinea pig bone marrow in homologous serum unheated and heated at 56°C. The growth of bone marrow was found to be more extensive in unheated than in heated serum. The differences in the action of both sera were more striking than in

[4] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397.

our experiments. This was due to the fact that Ingebrigtsen used a medium containing a very large amount of serum and observed lymphocytes instead of fibroblasts.

The increase of the inhibiting power of serum after it had been heated at 56° and 70°C. may be considered as due to the production, under the influence of heat, of a change which renders the serum more toxic for the homologous fibroblasts. But it may also be attributed to the destruction of substances presenting the same heat resistance as complement and amboceptor, and partly protecting the cells against the inhibiting action of a third substance resisting heat at 70°C. Serum modified by heat acts in an opposite manner on heterologous tissues. Heated serum is a better culture medium for heterologous cells than unheated serum, as has already been shown by Ingebrigtsen.[4] We have found lately that the inhibiting action of dog, rabbit, and cat serum, heated at 56° and 66°C., on the rate of multiplication of fibroblasts is very much decreased. It seems that the factors which protect the organism against foreign cells and bacteria might also oppose the growth-inhibiting factor of serum and allow the cells to display a greater activity.

CONCLUSIONS.

The inhibiting action of homologous serum on the proliferation of fibroblasts *in vitro* was increased after the serum had been heated at 56° and 70°C. This action decreased after the serum had been heated at 100°C.

CICATRIZATION OF WOUNDS.

XIII. THE TEMPERATURE COEFFICIENT.

BY ALBERT H. EBELING, M.D.

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INTRODUCTION.

Cicatrization is a complex phenomenon which probably requires the coordinated activity of many factors. Although it may be expressed by a simple formula,[1] its mechanism involves several different agencies. The formation of granulating tissue during the latent period,[2] its contraction, the mobilization and proliferation of the epithelium, and the wandering of the latter on the surface of the granulations are themselves intricate processes, which may be governed by physical as well as chemical changes. Alterations of the viscosity of the surface tension of the fluids and the anatomical structures may play as important a rôle as chemical transformations. In order to ascertain whether physical or chemical changes are more especially involved, the value of the temperature coefficient of the phenomenon was measured.

EXPERIMENTAL.

The experiments were performed on alligators because these animals may be kept as well at a temperature of 38° as at 23°C. Two young alligators were used, their weights being respectively 309 and 72 gm. A rectangular flap of skin on the ventral surface of the body was resected with a sharp knife and the outline of the wound traced with India ink upon a piece of cellophane placed over the surface. The tracings were transferred to paper and the areas measured with a

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[1] du Noüy, P. L., *J. Exp. Med.*, 1919 xxix, 329.

[2] Carrel, A., *J. Exp. Med.*, 1921, xxxiv, 425.

planimeter. The animals were placed in a room having a tempera-
ture of 38°C. until the wounds had healed. Several days later a second
resection of skin was made in a different area on the ventral surface
of the body of each animal, as nearly as possible the same size as the
first. This was done by transferring the outline of the previous
tracing to the skin surface and then excising the flap. The animals
were placed in a room, the temperature of which averaged 23°C.

The wounds could not be protected by a dressing. They were
necessarily slightly infected, and a membrane or scab formed at the
surface. Under this covering, cicatrization was taking place, but its
progress could not be followed closely. To overcome this hindrance
to precise observation, the edges of the scab were gently and progres-
sively loosened with the point of a knife and a pair of small thumb
forceps, and then the extent of the formation of the epithelial layer
under the scab could be seen. The membrane was easily loosened
from the portion of the wound which had already healed, while the

TABLE I.

Experiment No.	Weight of animal.	Temperature.	Area of wound.	Length of time required for cicatrization.	Temperature coefficient for 10°C.
	gm.	°C.	*sq. cm.*	*days*	
1	309	38	1.2	11	2.36
	314	23	1.3	29	
2	72	38	0.4	8	1.88
	75	23	0.5	19	

scab proved adherent wherever epithelization had not occurred. If
an attempt were made to remove the protecting membrane, a slight
hemorrhage started. As such a traumatism delays the process of
healing, observations had to be made with the greatest care. Errors
of 1 or 2 days in the time of cicatrization may have occurred on
account of the technical difficulties. Two experiments were made on
each animal, one at a temperature of 38°C., and the other at 23°C.
(Table I).

In the first experiment, the temperature coefficient obtained,
$\frac{K_1}{K_0}$, is equal to 2.47, and in the second to 1.90, for a difference of

15°C., between 38° and 23°C. The difference between the values of these two coefficients may be attributed to experimental errors, but is not of a greater magnitude than those observed in similar phenomena. Their value for a range of 10°C. (between 20° and 30°C.) was obtained by the formula quoted by Robertson:[3]

$$\frac{K_1}{K_0} = e^{\frac{\mu}{3}\left(\frac{T_1-T_0}{T_1 T_0}\right)}$$

In the first case, it is equal to 2.36, and in the second to 1.88 for the same interval. The mean value is 2.12.

DISCUSSION AND CONCLUSIONS.

For a rise of temperature of 10°C., the rate of cicatrization was increased about twofold. This result could be expected, since wound healing is closely related to the phenomenon of growth and regeneration. It is classical that changes in temperature affect the metabolism[4] and the development of certain organisms[5] in the same manner as a chemical reaction. In spite of the complexity of the factors which bring about the cicatrization of a wound, it appears that the velocity of the phenomenon depends on the rate at which certain chemical changes take place.

[3] Robertson, T. B., Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920, 419.

[4] Krogh, A., Z. allg. Physiol., 1914, xvi, 178.

[5] Hertwig, O., Arch. mikr. Anat., 1898, li, 319. Loeb, J., and Northrop, J. H., Proc. Nat. Acad. Sc., 1917, iii, 384. Moore, A. R., Arch. Entwcklngsmechn., 1910, xxix, 146.

ACTION OF ANTIGEN ON FIBROBLASTS IN VITRO.

BY ALBERT FISCHER, M.D.

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It is known that tissue cells cultivated outside of the organism are able to produce antibodies against a given antigen when it is added to the medium. Goat red blood corpuscles, added to the medium in which guinea pig bone marrow was cultivated, determined the production of hemolysins by the guinea pig cells.[1] In the experiments described in this paper, the action of an antigen on the rate of proliferation of fibroblasts was investigated. An attempt was made to learn whether one part of a fragment of culture would grow at the same rate as the other part, when a small amount of a foreign protein was added to the medium for a long period of time; and whether a change in their respective rates of growth would occur if the fibroblasts cultivated in homogenic plasma, and in the same plasma containing a small amount of a heterogenic protein, were transferred into a medium containing this latter protein under a high contentration.

Technique.

The tissues used in these experiments were taken from a 9 year old strain of fibroblasts.[2] The cultures were prepared in the usual way, incubated for 48 hours, measured by projection of the area of growth, and transferred to a fresh medium. The foreign protein used was human ascitic fluid; in a few experiments dog serum was used. The culture which was selected for the experiment was divided into two equal parts. One of the fragments was placed in a medium consisting of equal volumes of chicken plasma and fresh chick embryo juice. The other was placed in a medium containing equal volumes of plasma

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[1] Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

[2] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

and embryonic tissue juice to which had previously been added about 14 per cent of ascitic fluid, making the percentage of foreign protein in the total culture medium about 7 per cent. Care was taken to avoid contaminating the control culture by instruments, knives, and needles which had been in contact with the foreign protein. When transferred to a new medium, each of the two cultures was divided. One half was used for continuing the experiment, and the other half was discarded, unless this part was used as a test to see how the cultures would act when exposed to the foreign protein in a high concentration.

RESULTS.

The rate of growth of the two strains of a complete experiment of nineteen passages during a period of about 40 days is recorded in Table I and Text-fig. 1. At the beginning, the rate of growth of both cultures showed a slight difference. The culture to which the small amount of ascitic fluid was added nearly always showed a slightly higher rate of growth than the control culture. This might be due to secondary causes. The fluctuations of the rate of proliferation are due, to some extent, to the influence of slight changes in temperature of the incubator and similar periodical causes. When the control showed a high rate of growth, the experiment showed a lower rate, and *vice versa*, but after about eight or ten passages both cultures showed fluctuations in the same direction. As a whole, no marked differences between the two rates of growth seem to take place.

When an attempt was made to see how both strains would act when exposed to a high concentration of the protein used for immunization, subcultures were made of the control (non-immunized strain) and of the immunized strain, and transferred into a medium composed of plasma, embryonic tissue juice, and ascitic fluid. The amount of ascitic fluid in the subcultures was 50 per cent or more, and exerted a marked inhibiting influence on the rate of growth of a normal culture. The rate of growth of the control cultures which had not been exposed to ascitic fluid decreased markedly and was even followed by death (Text-fig. 2). At Passages 7, 10, 12, 14, and 17, half the tissue fragment was cultivated in a medium containing about 50 per cent of ascitic fluid, while the other half was used for the continuation of the

strain. The dots A, B, C, D, and E represent the rate of growth of these subcultures in 48 hours (Text-fig. 2.) It is shown that 50 per cent ascitic fluid has a marked inhibiting influence on the rate of

TABLE I.

Rate of Growth of the Immunized and Non-Immunized Strains of Fibroblasts for a Period of Nineteen Passages.

| Culture No. | Passage No. | Relative increase. | | Relative increase of subcultures in 50 per cent ascitic fluid. | | Ratio $\left(\dfrac{E}{C}\right)$ of subcultures. |
		Control; homogenic medium.	Experiment; homogenic medium with 7 per cent ascitic fluid.	Control.	Experiment.	
537-1, 537-2	1	7.0	10.2			
549, 550	2	9.8	6.2			
559, 560-1	3	9.4	14.0			
571, 572	4	5.6	8.2			
585, 586	5	9.0	16.8			
599, 600	6	12.4	13.4			
610-1, 610-2 } 611-1, 611-2 }	7	8.2	10.2	2.8	13.8	4.9
623, 624	8	7.6	15.8			
642, 643	9	17.0	19.0			
659-1, 659-2 } 660-1, 660-2 }	10	18.6	9.2	4.6	16.2	3.5
672, 673	11	23.9	17.2			
686-1, 686-2 } 687-1, 687-2 }	12	17.4	15.6	9.4	23.2	2.4
702, 703	13	19.0	20.8			
718-1, 718-2 } 719-1, 719-2 }	14	10.4	12.0	6.0	23.0	3.4
733, 734	15	17.4	15.8			
749, 750	16	12.4	6.8			
760-1, 760-2 } 761-1, 761-2 }	17	10.0	8.0	3.8	8.4	4.2
775, 776	18	13.2	10.4			
799, 800	19	9.0	12.2			

growth of a normal culture of fibroblasts. The rate of growth of the fibroblasts in a culture medium which always contained about 7 per cent ascitic fluid is shown in Text-fig. 3. At Passages 7, 10, 12, 14, and 17, subcultures were made in a medium containing about 50 per
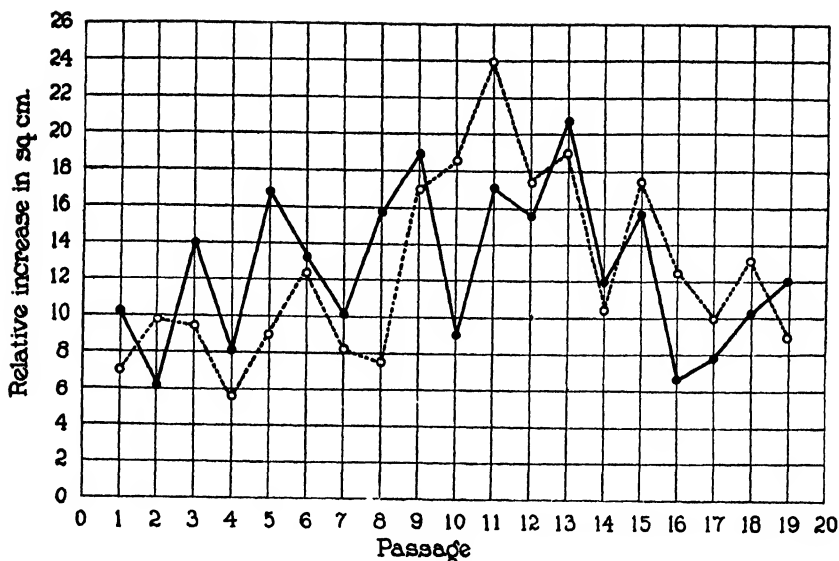
In all the figures the ordinates represent the relative increase in square centimeters and the abscissæ the number of passages at 48 hour intervals.

Text-Fig. 1. The rate of growth of two cultures of fibroblasts, the control (dotted line) cultivated in the usual homogenic culture medium, the other (solid line) in the same medium containing 7 per cent ascitic fluid.
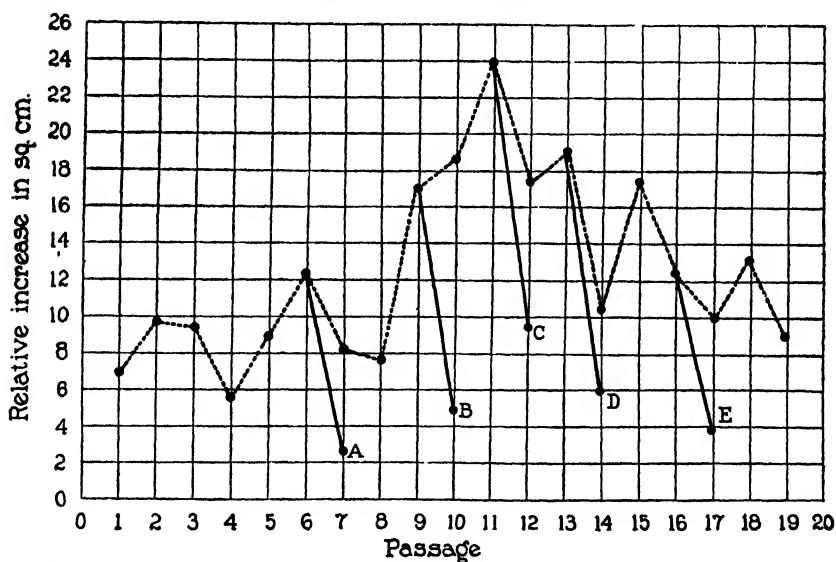
Text-Fig. 2. The rate of growth of the non-immunized strain and subcultures. The dotted line represents the rate of growth of fibroblasts in homogenic culture medium. The solid lines (A, B, C, D, and E) represent the rate of growth of the subcultures of the non-immunized strain in a medium containing 50 per cent ascitic fluid.

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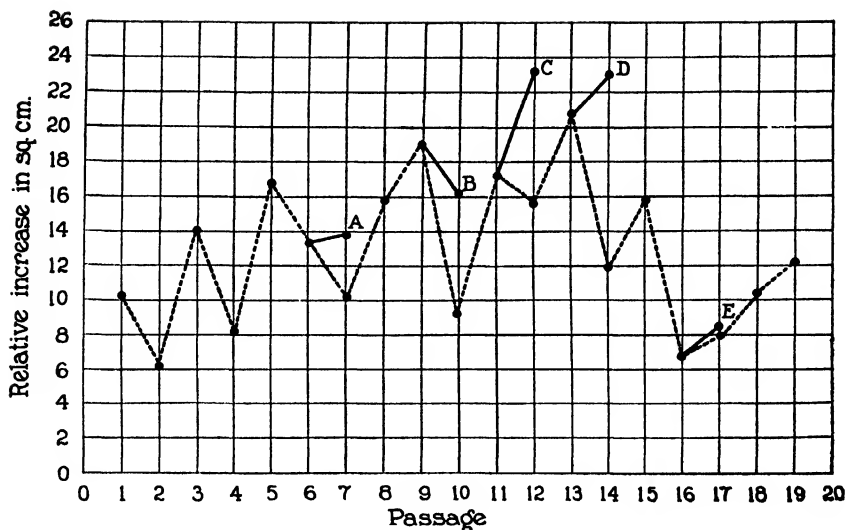
TEXT-FIG. 3. The rate of growth of the immunized strain and subcultures. The dotted line represents the rate of gowth of fibroblasts in the culture medium containing 7 per cent ascitic fluid. The solid lines (*A*, *B*, *C*, *D*, and *E*) represent the rate of growth of the subcultures of the immunized strain in a medium containing 50 per cent ascitic fluid.
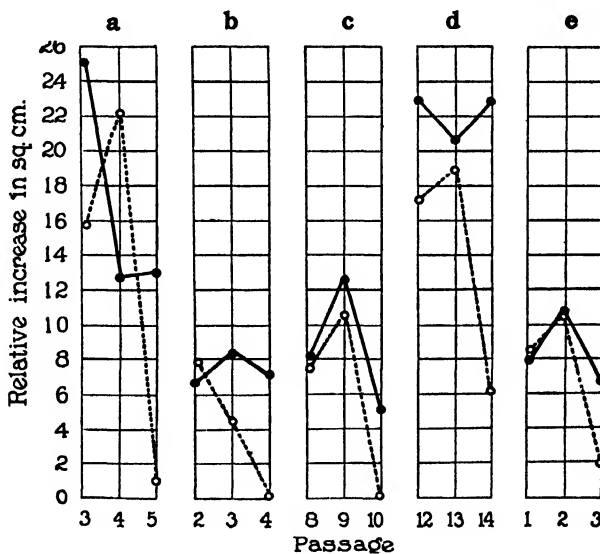
TEXT-FIG. 4, *a* to *e*. (*a* to *d*) The rate of growth of the non-immunized (dotted line) and the immunized (solid line) strain of fibroblasts. Of the three passages shown, the last one represents the rate of growth in the culture medium containing 50 per cent ascitic fluid (*e*) The rate of growth of two strains of fibroblasts, one (solid line) cultivated in a medium containing 7 per cent dog serum, the other (dotted line) in a medium containing no foreign protein. The last passage shows the growth of both strains in the culture medium containing 50 per cent dog serum.

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cent ascitic fluid, and the dots *A*, *B*, *C*, *D*, and *E* indicate the rate of growth. It may be seen that the rate of growth increased markedly instead of decreasing, as in the control cultures. There was always a sharp difference in the response of the two cultures to the same amount of foreign protein. It was certain'that one of the strains had become adapted to the presence of the foreign protein, while the other was still subject to its inhibiting influence. The same phenomena were observed in several other experiments and are expressed by Text-fig. 4, *a* to *e*. In Text-fig. 4, *a*, the curve shows the rate of growth during two passages in 7 per cent foreign protein, and after exposure to 50 per cent foreign protein. The non-immunized strain almost died. Its rate of growth decreased from 22 sq. cm. to about 1 sq. cm., while the immunized strain grew at the usual rate, as if nothing had happened. In another experiment (Text-fig. 4, *b*), the non-immunized strain was killed, and the immunized strain not affected at all. The same phenomenon is shown in Text-fig. 4, *c* and *d*.

In a few experiments, dog serum which had had a strong inhibiting influence on the growth of fibroblasts was used as antigen. The result showed the same action as with ascitic fluid (Text-fig. 4, *e*). The immunized strain was cultivated in a medium containing 7 per cent dog serum, and the control in a medium containing no foreign protein. For the final test, both strains were placed in a medium containing 50 per cent dog serum; the rate of growth of the control decreased much more than that of the immunized strain.

CONCLUSIONS.

It may be concluded that, under the conditions of the experiments:

1. A small amount of foreign protein added to the culture medium does not modify the rate of proliferation of fibroblasts.

2. A large amount of foreign protein added to the culture medium decreases markedly the rate of proliferation of fibroblasts cultivated previously in homogenic medium, while it does not decrease the rate of proliferation of fibroblasts cultivated previously in the presence of a small amount of the foreign protein.

3. Fibroblasts *in vitro* respond to the presence of an antigen in the culture medium by becoming immunized against its action.

A TEN YEAR OLD STRAIN OF FIBROBLASTS.

By ALBERT H. EBELING, M.D.

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PLATES 64 TO 66.

A strain of fibroblasts, obtained from the heart of a chick embryo on
January 17, 1912, has completed the 10th year of its life *in vitro*. On
January 17, 1922, our incubators contained about 60 cultures which
represented the 1860th generation of the connective tissue cells.
The purpose of this paper is to describe the actual condition of the
strain, to discuss its meaning, and to summarize the findings.

1. Present Condition of the Strain.—The growth of the tissue frag-
ments is as rapid today as during the past years. Each fragment
generally doubles its volume in 48 hours. The cultures (Fig. 1)
have not modified their appearance.[1–4] They are composed of typical
fibroblasts which grow in a dense tissue (Fig. 2) at the periphery of
which individual cells may be observed and photographed (Figs. 3
and 4). Many cells divide mitotically (Figs. 5 to 8).

·2. Unlimited Proliferation and Potential Immortality of Fibroblasts.—
The fact that fibroblasts have been kept in active condition for 10 years
has an important significance. It demonstrates that tissue cells
living *in vitro* transform the foodstuffs of their medium into proto-
plasm, and that their mass increases effectively. Some experimenters
believed that cells cultivated outside of the organism did not use the
substances contained in the medium,[5] and that the mass of the tissue
did not increase. This opinion applies to tissues cultivated in Locke

[1] Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

[2] Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273.

[3] Carrel, A., *J. Exp. Med.*, 1914, xx, 1.

[4] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277. Ingebrigtsen, R.,
J. Exp. Med., 1912, xvi, 421. Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335.
Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

solution, or by the plasma method, but not to tissues cultivated in a medium containing embryonic tissue juices.[6] Observation of our strain of fibroblasts shows this beyond all doubt. In 10 years, more than 30,000 cultures have been derived from a fragment of heart less than 1 c.mm. in size. If it had been feasible to multiply the tissues to their greatest possible extent, today their mass would be very much larger than the sun. Thus it is evident that some of the food material contained in the medium has been transformed into protoplasm.

The existence of the 10 year old strain demonstrates also that the cells are potentially immortal. This hypothesis was proposed long ago by Carrel when he observed that after 28 months of life *in vitro* the rate of growth of the active tissue cells had not decreased.[8] It was evident that fibroblasts living under the conditions of his experiments were no longer subjected to the influence of time, as they are when living within the organism. They could be compared to unicellular organisms, such as *Paramecium*, which Woodruff has cultivated for over 14 years,[7] and could be considered as potentially immortal. This opinion was criticized by Harrison,[8] who considered it unjustified until the cells should have lived in cultures for a period exceeding the duration of life of the organism from which they were taken. Pearl[9] believes that the present age of the strain of fibroblasts fully disposes of this criticism, as the limit of the life of chickens is often 10 years. It may be considered as certain, therefore, that fibroblasts will proliferate indefinitely, as do colonies of Infusoria.

3. *Use of the Strain of Fibroblasts in Physiological Studies.*—The purpose of the experiments was not only to ascertain whether fibroblasts could proliferate indefinitely, but also to study certain biological problems. Pure cultures of cells[10] are as necessary in physiology as pure cultures of microorganisms in bacteriology. The strain, being composed only of typical fibroblasts, grows with great regularity, and was found to respond readily to changes in the composition of the culture medium by a modification of its rate of proliferation. The

[6] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.
[7] Woodruff, L. L., cited by Pearl, R., *Scient. Monthly*, 1921, xii, 202.
[8] Harrison, R. G., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 71.
[9] Pearl, R., *Scient. Monthly*, 1921, xii, 332, 333.
[10] Carrel, A., *J. Exp. Med.*, 1912, xvi, 165.

original technique[1,11] was progressively perfected. Studies of the optimum medium from the point of view of osmotic tension[12] and H ion concentration[13] were made. Substitution of a fibrinogen suspension for plasma was rendered possible.[14] More accurate methods of preparing and measuring the cultures were developed,[15] by which differences of less than 10 per cent in the rate of growth could be ascertained.

The strain, then, could be used as a reagent for the detection of substances contained in the humors which have the power of activating or decreasing the rate of cell proliferation. The variations of the rate of growth demonstrated the presence in embryonic juice of a factor which increases the velocity of cell multiplication to a high degree.[6,16] It showed also that the state of the fibroblasts at a given time was function of two independent variables, their dynamic condition at the preceding instant, and the condition of the medium.[17] It became evident that the activity of the fibroblasts does not depend on the amount of potential energy they contained at the beginning of their life but upon certain substances present in the medium. The cells remained indefinitely young or grew old[17] according to the food material they were given and the extent of the elimination of their catabolic substances.

In the same manner, it was found that adult serum produces on fibroblasts an effect opposite to that of embryonic tissue juice. This inhibiting power of a serum increased very much with the age of the animal from which it was obtained.[18] As there was a definite relation between the age of the animal and the rate of cell proliferation in its serum, the growth index of the serum indicated the variations in the age.[19]

[11] Carrel, A., *J. Am. Med. Assn.*, 1911, lvii, 1611.

[12] Ebeling, A. H., *J. Exp. Med.*, 1914, xx, 130.

[13] Fischer, A., *J. Exp. Med.*, 1921, xxxiv, 447.

[14] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

[15] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

[16] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

[17] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

[18] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxv, 17.

[19] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

The strain was used also in the study of the response of tissue cells to an antigen. It is known that an antigen added to a culture of bone marrow determines the formation of an antibody.[20] When the strain was sensitized by a foreign protein, its response to a second injection[21] showed that it could be used in the investigation of certain phenomena of immunity. As it has become possible lately to obtain strains of lymphocytes[22] and of epithelial cells[23] living *in vitro* by practically the same procedure that is used for fibroblasts, the scope of these studies will be increased.

EXPLANATION OF PLATES.

PLATE 64.

FIG. 1. Culture 24871–1. Passage 1858. 10 years, lacking 4 days, of life *in vitro*. Portion of the central and peripheral zone. Among the peripheral cells, a number of mitoses can be distinguished. Stained with methylene blue after 48 hours growth. × 100.

PLATE 65.

FIG. 2. Culture 24870–1. Passage 1858. 10 years, lacking 4 days, of life *in vitro*. Very active culture. Peripheral area in which nine mitotic figures can be observed. Stained with methylene blue after 48 hours growth. × 300.

PLATE 66.

FIG. 3. Culture 24871–1. Passage 1858. Typical fibroblast in the peripheral zone of the culture shown in Fig. 1. × 1,000.

FIG. 4.[24] Culture 422. Passage 1710. 9 years, 3 months, lacking 4 days, of life *in vitro*. Typical fibroblast. Stained with gold chloride after 48 hours growth. × 1,100.

FIGS. 5 and 6. Culture 24041–2. Passage 1844. A cell undergoing mitotic division after 10 years, lacking 1 month, of life *in vitro*. Stained with methylene blue after 48 hours growth. × 1,000.

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[20] Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

[21] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 661.

[22] Carrel, A., and Ebeling, A. H., unpublished experiments.

[23] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

[24] I am indebted to Dr. Albert Fischer for permission to show a picture of this particular preparation.

(Ebeling Ten year old strain of fibroblasts.)

(Ebeling Ten year old strain of fibroblasts)

(Ebeling: Ten year old strain of fibroblasts.)

FIG. 7. Culture 964 A. Passage 1857. 10 years, lacking 2 days, of life *in vitro*. Fibroblast a short time after division. Stained with methylene blue after 48 hours growth. × 1,000.

FIG. 8. Culture 964 A. Passage 1857. Double mitoses in an actively growing culture, after 10 years, lacking 2 days, of life *in vitro*. Stained with methylene blue after 48 hours growth. × 1,000.

QUANTITATIVE LAWS IN REGENERATION.

III. The Quantitative Basis of Polarity in Regeneration.

By JACQUES LOEB.

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I. INTRODUCTION.

It has been shown in preceding papers that the dry weight of shoots and roots produced under equal conditions of illumination, moisture, and temperature in sister leaves of *Bryophyllum calycinum* varies approximately in direct proportion with the dry weight of the leaves; and that the same is true for the mass of shoots produced in small pieces of stem connected with a leaf.[1] It had been known that when a piece of stem is left in connection with a leaf, the mass of shoots produced by the leaf is less than when the leaf is completely isolated, and the writer had been able to show that in this case the stem connected with the leaf gains approximately as much in dry weight as the dry weight of the shoots and roots in the leaf would have been if the leaf had been completely isolated from the stem.[2] The inhibitory influence of the stem on the shoot and root formation in the leaf was in this case due to the fact that when the leaf is connected with a stem, that part of the material which could have been utilized for the formation of new shoots and roots in the leaf now goes into the stem. It is intended to show in this paper that the same simple quantitative relations suffice to account for the polar character of regeneration in a defoliated stem of *Bryophyllum*.[3]

The reader will remember that each node of the stem of this plant has two dormant buds capable of growing into shoots. When a piece of defoliated stem is cut from a plant and suspended in moist air, only

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[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 81; 1919–20, ii, 297, 651. *Science*, 1917, xlv, 436. *Bot. Gas.*, 1918, xlv, 150. *Ann. Inst. Pasteur*, 1918, xxxii, 1.

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 297, 651.

[3] Loeb, J., *Science*, 1921, liv, 521.

the two buds of the most apical node will grow into shoots, while the buds in all the nodes below will remain dormant.   Permanent roots will grow only at the base of each piece, though transitorily air roots

In air
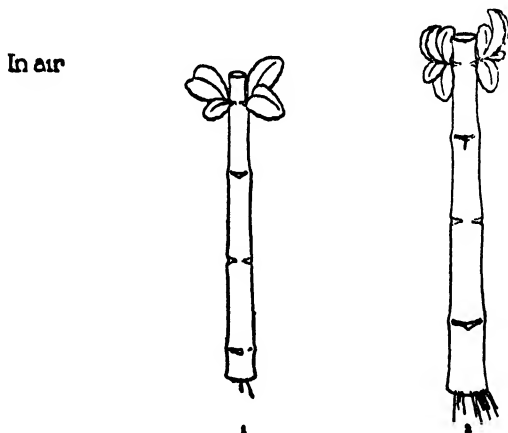
FIG. 1. Pieces of stem from the same plant, (1) apical, (2) basal. Suspended in moist air, shoots formed only in the apical node, roots at the base. Mass of shoots and roots is larger in the basal piece (2) which has the larger mass. Duration of experiment October 4 to November 7, 1921.

In air
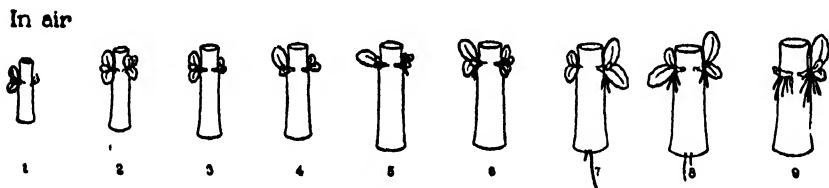
FIG. 2. Stem cut into small pieces with 1 node each. Suspended in same aquarium and simultaneously with large stems in Fig. 1. (1) was the most apical, (9) the most basal piece, the serial number denoting the original position of the pieces in the plant. Each piece of stem forms 2 shoots in its node, but the relative mass of the shoots varies with the relative mass of the stem, not with the serial number of the node.

may begin to form in any node, but these will dry out as soon as the basal roots are growing.[4] Fig. 1 illustrates this polar character of regeneration in defoliated pieces of stem suspended in moist air. When, however, a long defoliated stem is cut into as many

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 687.

pieces as there are nodes, then all the dormant shoot buds of the stem will grow out into shoots (Fig. 2). The stems in Figs. 1 and 2 were cut out at the same time and suspended in moist air in the same vessel.

The results remain about the same when the basal ends of the pieces are dipped into water, the only difference being that often not only the
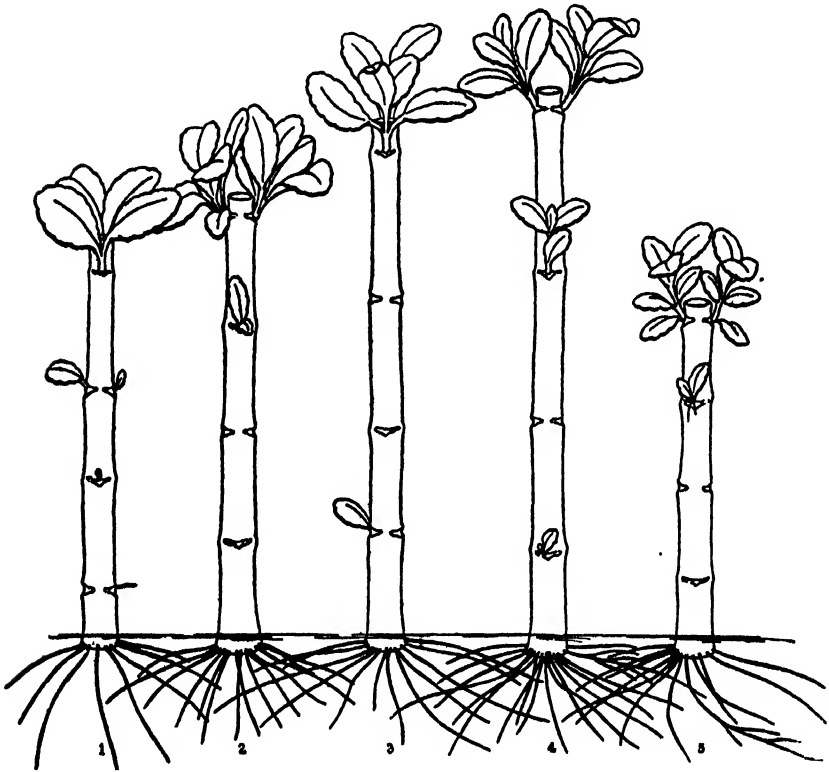
FIG. 3. Same experiments as Fig. 1, only that the long pieces of stem were put with their bases into water. Duration of experiment from September 27 to October 22, 1921. All stems were cut from one plant.

two buds in the most apical node of a long piece of stem grow out but also one or two buds of the node below (Fig. 3). The amount of growth of shoots and roots is also greater in the stems put with the base in water (Fig. 3) than when the stems are suspended in moist air (Fig. 1). When pieces of stem with only one node each are put into water, each piece forms shoots at its node (Fig. 4). The

question is, Why do only the most apical buds of a long defoliated stem grow out?   Bonnet had suggested that the ascending sap of a plant was shoot-producing and the descending sap was root-producing.   Sachs pointed out that when a piece of stem was cut out from a plant the ascending sap was blocked at the apex and that hence the shoot-producing substances must collect at that end of the stem giving rise to shoots at the apical node; while the descending sap is blocked at the base, giving rise to root formation at that end.

The problem then exists to prove or disprove the old suggestion of Bonnet and Sachs.   The formation of shoots or roots is a synthetic process or a series of catenated synthetic processes, in which soluble
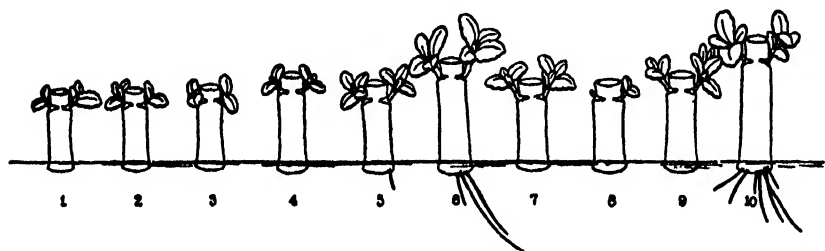
Fig. 4. Stem of one plant cut into ten small pieces, the serial number indicating their position in the plant, (1) being the most apical, (10), the most basal piece. Base in water.   Experiment simultaneous with Experiment III.   Each piece has formed 2 shoots the relative size of which does not follow the serial number of the stem, but the relative size.   The size of each shoot of the pieces is much smaller than the size of the shoots formed simultaneously by the larger stems in Fig. 3.   The latter stems all have roots, while only the two largest pieces of stems (6) and (10) in Fig. 4 have formed roots.

materials, such as sugars, amino-acids, and other substances, are synthetized into the larger molecules of proteins, compounds of the cellulose type, and others.   If the theory of Bonnet and Sachs is correct, it must be possible to show that the two shoots formed at the apex of a long defoliated piece of stem have, within the limits of the accuracy of the experiments, approximately the same dry weight as the dry weight of all the shoots would have amounted to if the stem had been cut into as many small pieces as it contained nodes.

By comparing the amount of shoots formed in the one-node pieces in Fig. 2 or 4 with those of the four-node pieces in Fig. 1 or 3, the reader will notice that the shoots are greater in the larger pieces of

stem, and the same fact is obvious from all the other figures in this paper. It is almost obvious from a glance at the figures that the mass of shoots formed increases with the mass of the stem. If the mass of shoots produced at the apex of large pieces of stem is approximately equal to the mass of shoots which the same stems would have produced had they been cut into as many pieces as the stems contained nodes (*i.e.*, into one-node pieces), it will be necessary to show that within the limits of the experimental errors, the mass of dry weight of shoots produced per gram of dry weight of stem is about the same regardless of whether the stems are long or whether they are subdivided into one-node pieces.

This was tested in various ways. The defoliated stem of a very large plant was cut into 5 pieces, each possessing 4 nodes (Fig. 3), and the defoliated stem of a second plant was cut into 10 small pieces of 1 node each (Fig. 4). The pieces dipped with the base into water and the large and small pieces were suspended in the same aquarium. The experiment lasted from September 27 to October 22, 1922. The shoots were then cut off and both shoots and stems were dried for 24 hours in an oven at about 100°C. The result was as follows: The dry weight of the 5 large stems (Fig. 3) was 13.670 gm., and the dry weight of their 16 shoots was 0.495 gm. The shoot production was therefore 36 mg. per gram of stem (all measured in dry weight). The dry weight of the 10 short pieces of stem with 1 node each (Fig. 4) was 2.880 gm., and the dry weight of 19 shoots was 0.115 gm., or 1 gm. of dry weight of stem produced 40 mg. of dry weight of leaves. These two figures, 40 mg. and 36 mg., agree sufficiently closely to show that under equal conditions the production of shoots of defoliated pieces of stem occurs in proportion with the mass of the piece of (defoliated) stem; or, in other words, the mass of shoots produced at the apex of the large defoliated stems of Fig. 3 is approximately equal to the mass of shoots the same stems would have produced if all the dormant buds of each stem had been able to grow out.

The experiment in Figs. 1 and 2 gave a similar result. The experiment lasted from October 4 to November 7. 5 large stems with 4 nodes each (Fig. 1) having a dry weight of 5.486 gm. produced 10 shoots with a dry weight of 0.114 gm.; *i.e.*, 20.8 mg. of shoot per gram of stem.

4 short pieces of stem with 2 nodes each, having a dry weight of 3.214 gm., produced 8 shoots with a dry weight of 0.0668 gm.; *i.e.*, 20.7 mg. of shoot per gram of stem.

A third stem was cut into 9 pieces with 1 node each (Fig. 2) possessing a dry weight of 3.270 gm., giving rise to 17 shoots with a dry weight of 0.050 gm.; *i.e.*, 15.3 mg. of shoot per gram of stem.

The first two figures are identical, the last figure is a little low. In these experiments the end of the piece may suffer (by drying out or falling a prey to fungi) and this creates an error which is especially noticeable when a stem is cut into many small pieces. But in spite of these sources of error the results are remarkably clear and consistent.

It seemed of interest to compare the behavior of defoliated stems split longitudinally. In this case the two halves should give approximately equal results.

II. *Experiments with Split Stems.*

Experiments were made with stems split longitudinally as indicated in Fig. 5. Only pieces from the middle of the stem of a large plant were used, for reasons to be given later. Stems with 4 nodes each, were split longitudinally and one half was cut transversely into 2 pieces with 2 nodes each, *a'*, *b'*, and *c'*, *d'*, respectively (see Fig. 5). The other half with the 4 nodes *a*, *b*, *c*, and *d* was not cut transversely. All 3 pieces (Fig. 5) were put with their bases into water. It was to be expected that the sum of the dry weight of the shoots produced by the 2 small pieces with 2 nodes each should equal the dry weight of the shoots produced by the larger pieces with 4 nodes each. Fig. 5 shows at a glance that this is approximately the case and the dry weight determinations confirm this.

The first experiment was carried out on 7 stems, a second experiment on 16 stems. Table I gives the result.

It is therefore obvious that the dry weight of the sum of the shoots produced by the small pieces *a'*, *b'*, and *c'*, *d'*, approximately equals the dry weight of the shoots produced by the big pieces, *a*, *b*, *c*, and *d* (Fig. 5), or, in other words, the mass of shoot produced at the apex of the large pieces is approximately equal to the dry weight of the shoots the same stems would have produced if the buds of every second node had been able to grow.

TABLE I.

Experiment No.	Duration of experiment.	Number of pieces.	Dry weight of shoots produced.	Dry weight of stems.	Shoots produced per gram of stem.
	1921		*gm.*	*gm.*	*mg.*
I	Nov. 3– Dec. 6.	7 four-node pieces, *a*, *b*, *c*, *d*.	0.1545	4.290	36.0
		14 small pieces, *a'*, *b'*, *c'*, *d'*.	0.147	3.822	38.7
	1921-22				
II	Dec. 8– Jan. 10.	16 four-node pieces, *a*, *b*, *c*, *d*.	0.750	16 646	45.0
		32 two-node pieces, *a'*, *b'*, *c'*, *d'*.	0.577	14.527	39.5
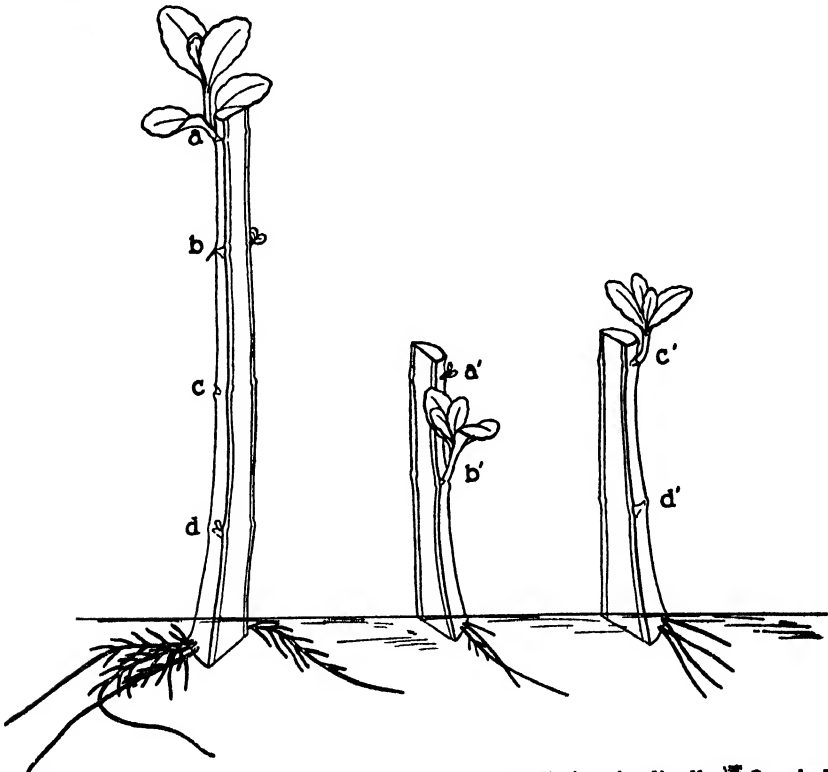
FIG. 5. Piece of stem with 4 nodes, *a*, *b*, *c*, *d*, split longitudinally. One half cut transversely into two pieces, *a'*, *b'*, and *c'*, *d'*. The half *a*, *b*, *c*, *d*, produces 1 shoot which about equals in mass the 2 shoots produced by *a'*, *b'*, and *c'*, *d'*. Duration of experiment December 9, 1921, to January 4, 1922.

III. *Small and Large Pieces of the Same Stem.*

A third series of experiments was 'as follows: Long pieces of stem, containing about 10 nodes, were cut out from the same plant more
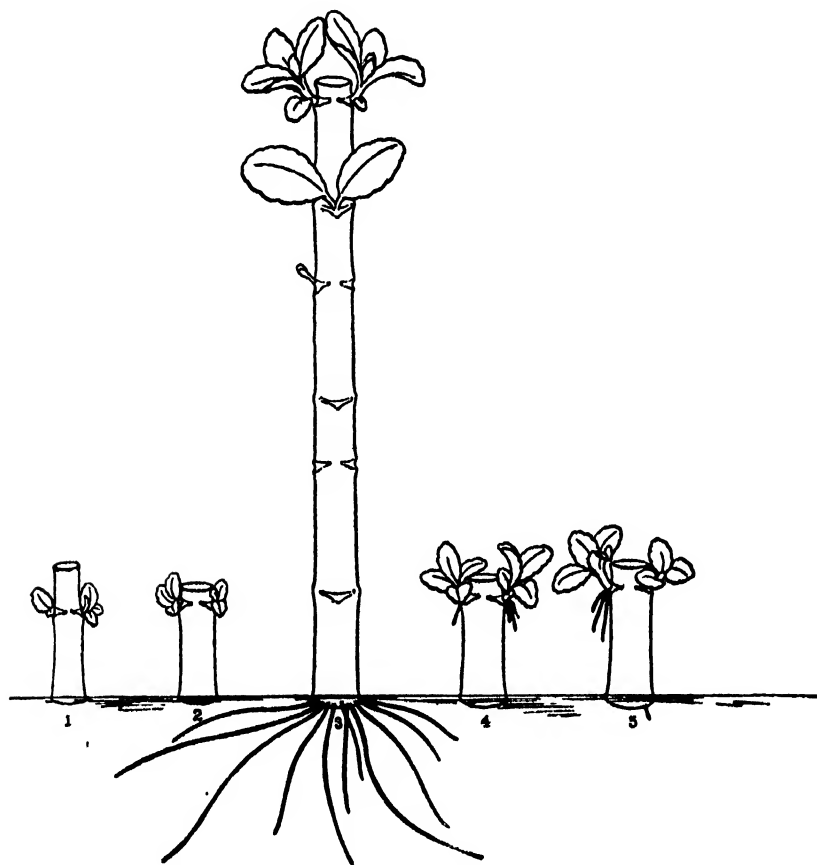
FIG. 6. Five pieces cut from the stem of the same plant, (1) and (2) apical pieces with 1 node each, (3) middle piece with 6 nodes, and (4) and (5) the basal pieces with 1 node each. The large middle piece produces larger shoots than either the more apical or more basal small pieces. The large middle piece has ample roots while only the longer basal piece commences to form a root. Duration of experiment October 25 to November 21, 1921.

than 1 year old (Fig. 6).   The middle piece of about 6 nodes (piece 3 in Fig. 6) served for the experiment, two small pieces, 1 and 2, containing 1 node each and situated apically, and 2 small pieces, 4 and 5, also containing 1 node each, situated basally from the large middle

piece in the same stem, serving as controls.   In other experiments of
the same character, pieces containing about 14 nodes were cut out
from the stem of the same plant; 2 small pieces at the apex, each con-
taining 2 nodes (1 and 2, Fig. 7), and 2 small pieces at the base each
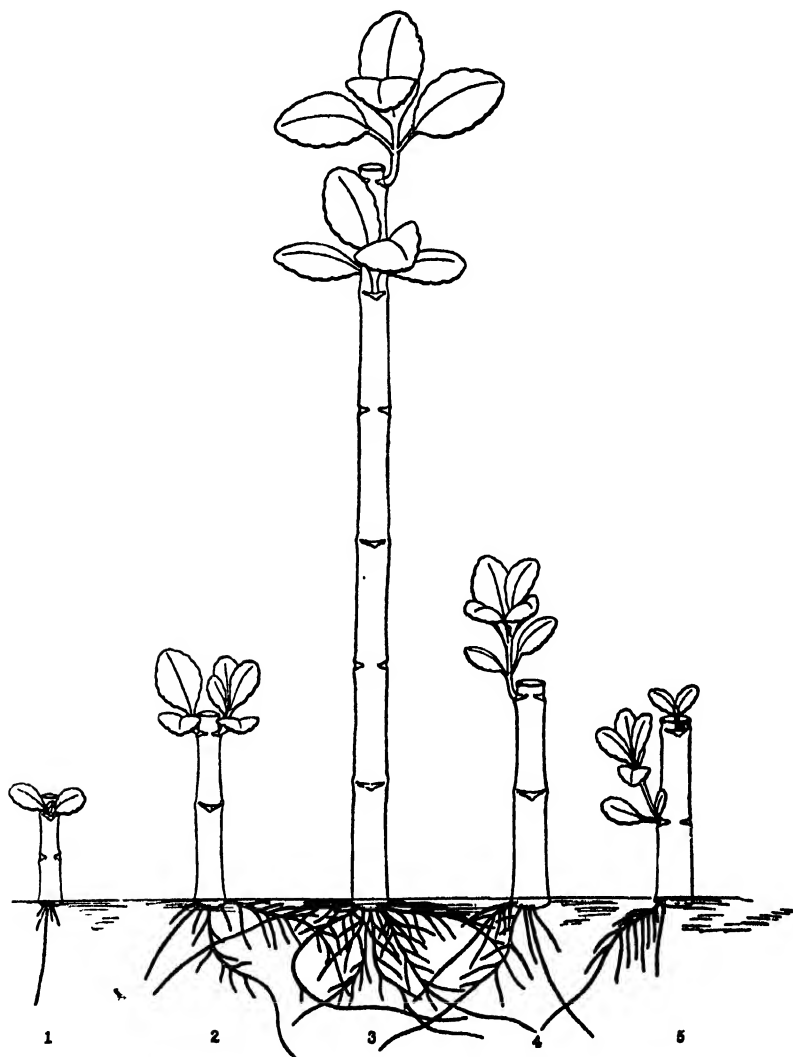
FIG. 7. Similar experiment as Fig. 6, except that the small pieces have 2 nodes
each.   Shoots and roots are formed in proportion to mass of stem.   Duration of
experiment November 16 to December 19, 1921.

containing also 2 nodes (4 and 5, Fig. 7), were used as controls, while the middle piece (3, Fig. 7) served for the main experiment. All the pieces dipped with their bases into water.

It is obvious from Fig. 6 that the large pieces of stem (3) produced larger masses of shoots than the small pieces 1 and 2 or 4 and 5 during the same time and under equal conditions. It may also be pointed out that these large middle pieces (3) formed their basal roots earlier than the small pieces (Fig. 6), and that the mass of their roots remained greater than the mass of roots in the small pieces (Fig. 7).

It turned out that the shoot production in the most apical pieces of stems 1 and 2 was usually irregular, as a rule too small, so that these pieces were not well usable as controls. The basal pieces, 4 and 5, however, behaved normally. It seems that this abnormal behavior of the small apical pieces is found as long as the leaves connected with this piece are still small and growing. It is therefore well to use in these experiments that part of the stem which is naturally defoliated or the leaves of which are about to fall. It may also be well not to use pieces of stem too near the roots. After 3 to 5 weeks the dry weight of the shoots and of the stem used in these experiments were determined. Since some of the small pieces of stem fall often a victim to fungi only one of the 2 small pieces, apical or basal, was used as a control.

Experiment I. October 25, 1921, to November 25, 1921.

		Dry weight of shoots per gram of stem.
6 long pieces with 6 nodes each.	*gm.*	*mg.*
Dry weight of stems.........................	9.260	
" " " 13 shoots.....................	0.260	28 0
" " " roots.........................	0.057	
Control a. 7 short basal pieces with 1 node each.		
Dry weight of stems.........................	2.895	
" " " 13 shoots..........	0 088	30 4
" " " roots.........................	0 003	
Control b. 12 short apical pieces with 1 node each.		
Dry weight of stems.........................	1.428	
" " " 18 shoots.....................	0.0236	16.5

It is obvious that the apical control pieces gave too small a production of shoots (16.5 mg. per gram of stem), while the basal con-

trol pieces produced approximately the same amount of shoots per gram of stem, namely 30.4 mg. as compared with 28.0 for the large pieces.

Experiment II. November 2, 1921, to December 6, 1921.

		Dry weight of shoots per gram of stem.
5 long pieces of stem with 6 nodes each.	gm.	mg.
Dry weight of stems......................... 6.486		42.0
" " " 10 shoots..................... 0.272		
" " " roots........................ 0.0458		
Control a. 4 short basal pieces with 1 node each.		
Dry weight of stems........ .... 1.058		39.0
" " " 8 shoots................. .. .. 0.041		
" " " roots........................ 0 0034		
Control b. 5 short apical pieces with 1 node each.		
Dry weight of stems......................... 0.544		33.0
" " " 10 shoots................. ... 0.018		

Again the short basal control pieces produce about as much shoot material per gram (39 mg.), as the large pieces (42 mg.), while the apical controls produce less, namely, 33 mg. We will omit the apical controls in the further tabulation of experiments on account of the irregularity of the results.

Experiment III. November 16, 1921, to December 20, 1921.

		Dry weight of shoots per gram of stem.
9 long pieces of stem with 6 nodes each.	gm.	mg.
Dry weight of stems.........................18.658		50.3
" " " 26 shoots..................... 0.944		
" " " roots........................ 0.1428		
Control. 18 small basal pieces of 2 nodes each.		
Dry weight of stems.........................18.147		44.0
" " " 36 shoots..................... 0.800		
" " " roots........................ 0.136		

Experiment IV. October 22, 1921, to November 15, 1921.

4 long pieces of stem with 4 nodes each.		
Dry weight of stems......................... 4.214		21.0
" " " 8 shoots..................... 0.089		
Control. 4 short basal pieces of 2 nodes each.		
Dry weight of stems......................... 2.492		19.0
" " " 8 shoots..................... 0.0475		

Experiment V. October 11, 1921, to November 1, 1921.

		Dry weight of shoots per gram of stem.
	gm.	*mg.*
4 long apical stems with 6 nodes each.		
Dry weight of stems........................ 3.921		
" " " 8 shoots...................... 0.113		29.0
" " " roots........................ 0.0134		
Control. 4 basal pieces of 2 nodes each.		
Dry weight of stems........................ 3.744		24.0
" " " 10 shoots.................... 0.090		

Experiment VI. December 11, 1921, to January 17, 1922.

7 long apical stems with 6 nodes each.		
Dry weight of stems........................ 6.634		
" " " 12 shoots.................... 0.340		51.0
" " " roots........................ 0.0512		
Control. 7 short basal pieces of 2 nodes each.		
Dry weight of stems........................ 3.560		
" " " 12 shoots.................... 0.1770		49.6
" " " roots........................ 0.0128		

If we consider only those figures in the experiments where the small control pieces of stem were situated basally from the long stem (the pieces 4 and 5 in Figs. 6 and 7), we notice that the differences of shoots produced per gram of dry weight of the controls differ never more than 25 per cent from those produced by the large pieces of stem and that in some cases the difference is only about 6 per cent.   Considering the limitations in the experimental conditions—especially the fact that part of the stem may not function normally, especially the ends near the cut, or the fact that individual buds may have been injured by parasites, etc.—the agreement of the figures seems remarkable.

These results leave no doubt that within the limits of accuracy of these experiments the dry weight of the shoots produced at the apex of a long piece of defoliated stem is about equal the mass of shoots the same stem would have produced had the buds in all of its nodes been able to develop.

IV. Regeneration of Roots.

Two kinds of roots are formed in an isolated piece of stem, suspended in moist air and dipping with the base in water, first, air roots in the nodes, and later roots at the basal end of the stem regardless of the node (Figs. 1, 3, and 7). The air roots in the nodes grow out sooner than the basal roots but as soon as the basal roots grow out the air roots dry out and die. This has been discussed in a previous paper. We are interested here only in the basal roots since they alone are connected with the problem of polarity. The regeneration of the basal roots differs from the formation of apical shoots in this, that the apical shoots begin to grow out almost immediately after the defoliated piece of stem is isolated, while there is a long latent period before the basal roots make their appearance. For this reason quantitative measurements correlating the mass of the basal root formation with the mass of stem require probably a longer time than that in our experiments. A glance at the drawings will, however, convince the reader that the root formation commences sooner in the stems with larger mass than in the stems with smaller mass, regardless of the original position of the piece of stem in the plant.

Thus in Fig. 6 the large middle piece (3) forms roots before either of the 2 more basal pieces form roots, and Fig. 7 shows that the relative mass of roots produced seems also to run parallel with the relative mass of the piece. The same phenomenon is shown if we compare Fig. 1 with Fig. 2, or Fig. 3 with Fig. 4. It is also obvious in Fig. 5, so that we can say that the mass of roots produced by pieces of defoliated stem of *Bryophyllum calycinum* increases under equal conditions with the mass of the stem.

V. Influence of Light on Regeneration in a Defoliated Stem.

8 long defoliated stems were suspended into an aquarium kept dark by a double cover of black cardboard, and 8 equally long defoliated stems were put at the same time into an aquarium exposed in the usual way to daylight. The base of the stems dipped into water. All conditions were equal except the illumination. After 23 days all the stems exposed to light had formed large basal roots and large shoots at the apex (Fig. 8). At the same time none of the stems

Dark　　　　　　Light
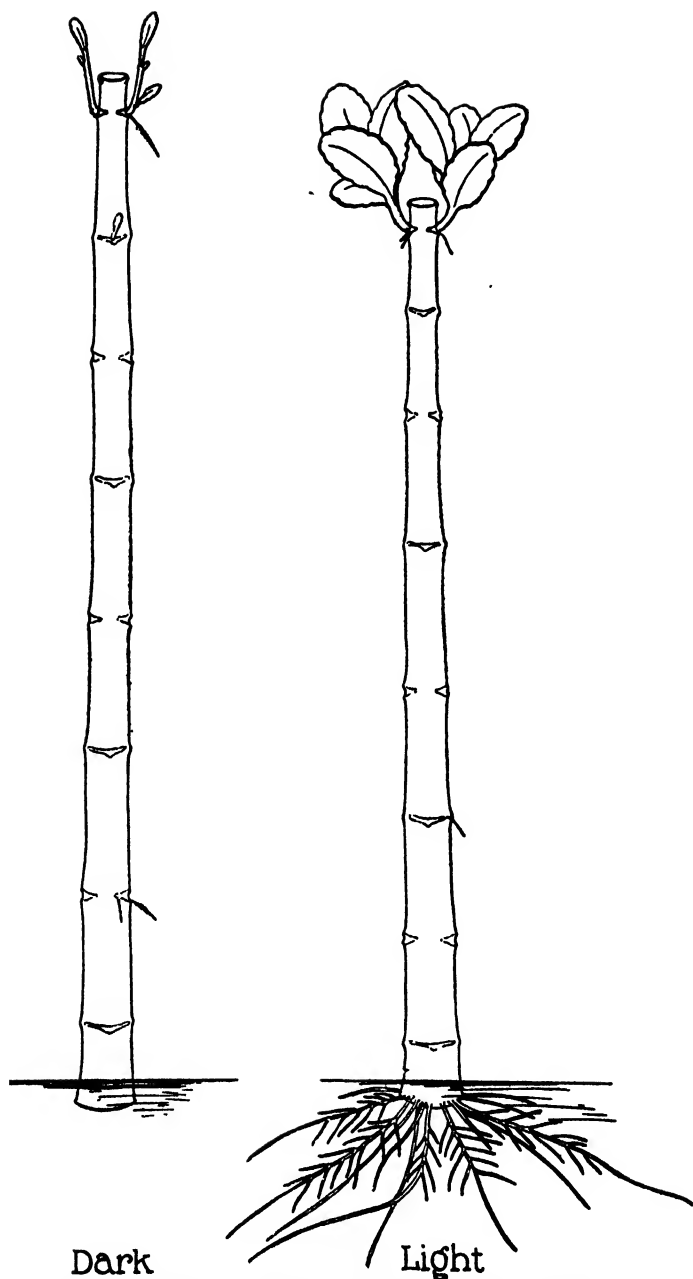
FIG. 8. Influence of light on root and shoot formation of stem.　In the dark no roots are formed; in light ample roots are formed.　The mass of shoots formed in dark is small compared with mass of shoots formed in light.

in the dark had formed a single basal root though some had formed tiny air roots (Fig. 8).   The shoots formed in the dark had a small mass and the typical etiolated shape.   The most striking phenomenon was the lack of root formation at the base of the stem in the dark. The writer had already shown that the favorable influence of the leaf on root formation in the stem also disappears when the leaf is deprived of light.[4]

SUMMARY AND CONCLUSION.

It is well known that a long defoliated piece of stem of *Bryophyllum calycinum* forms shoots only at the apical or the two apical nodes. while when such a stem is cut into as many pieces as there are nodes each node produces shoots.   It is shown in this paper that the dry weight of shoots produced in the apical nodes of a long piece of stem is approximately equal to the dry weight of shoots the same stem would have produced if it had been cut into as many pieces as it possesses nodes.   Hence all the material which can be used for the growth of shoots goes into the most apical part of the stem and this accounts for the polar character of regeneration in this case.[5]

It seems that the mass of basal roots produced by a piece of defoliated stem also increases with the mass of the stem.

[5] A plant morphologist, to whom the writer showed these experiments, commented that he was convinced that the shoot formation of an isolated piece of stem was due to a "stimulus."   If we accept this suggestion, it follows that the "stimulus" for regeneration must have varied quantitatively with the mass of the defoliated stem in our experiments, and this would lead us again to the idea that the "stimulus" must be something material since it cannot well be spiritual.

ELECTRICAL CHARGES OF COLLOIDAL PARTICLES AND ANOMALOUS OSMOSIS.

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(Received for publication, January 26, 1922.)

I. *The Transport Curves on the Acid Side of the Isoelectric Point of the Membrane.*

The experiments described in a preceding paper[1] leave no doubt that the Donnan equilibrium is the main source of the potential differences between solid gelatin particles and the surrounding liquid. On the other hand, experiments on the influence of salts on electrical endosmose, cataphoresis, anomalous osmosis, and Quincke's current potentials suggest in certain cases at least a second source which is generally designated as adsorption potentials. The difference between the two kinds of potentials should be that while the potential differences due to the Donnan equilibrium depend on the ionization of the protein, the adsorption potentials should occur regardless of whether or not the solid colloid is ionized. Adsorption potentials should, therefore, be found just as well in the case of isoelectric protein where the protein is practically non-ionized as in the case of metal proteinates or protein-acid salts, while the P.D. due to the Donnan equilibrium should be restricted to the latter two forms of protein.[2]

It is intended to investigate on the basis of this idea whether or not there exist at the surface of solid gelatin adsorption potentials in addition to potentials due to the Donnan equilibrium. It will be necessary to use for this purpose either electrical endosmose or anomalous osmosis or Quincke's currént potentials or cataphoresis. We shall select in this paper anomalous osmosis. By anomalous osmosis

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 351.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351.

is meant the superposition of electrical forces over the purely osmotic forces in the transport of water through a membrane separating pure water from a solution of an electrolyte (or separating two different solutions of electrolytes). When both water and electrolytes are capable of diffusing through the membrane the difference in the mobility of the oppositely charged ions will cause diffusion potentials acting across the membrane. In this case the solution assumes the opposite sign of charge as the water. These potentials we will call E.

There may be a second P.D. inside the pores of the membrane between the solid wall of the pore and the liquid inside the pore. This potential we will call ϵ. If as the consequence of ϵ the liquid inside the pore assumes a negative charge, while as a consequence of E the solution in the collodion bag assumes a positive charge, the liquid cylinder inside the pore will be dragged into the solution by these electrical forces and thus an electrical transport of water will be added to the transport of water by osmotic forces. If the solution, however, has the same sign of charge as the liquid inside the pore, the electrical force will act in an opposite sense from the osmotic force, and the flow of water from the water side of the membrane into the solution will be less than is to be expected on the basis of van't Hoff's law.

This theory of anomalous osmosis was first suggested by Girard[3] and has later been supported by Bartell[4] and others.

In the experiments to be described salt solutions of a definite pH but of different concentrations were put into collodion flasks of about 50 cc. volume, which had received a coating of gelatin as described in previous papers. The collodion bags were dipped into 350 cc. of water of the same pH as that of the salt solution, but containing no salt. The collodion bags were closed with a rubber stopper perforated by a glass tube serving as manometer. The temperature was 24°C. and the rise of the liquid in the manometer was read 20 minutes after the commencement of each experiment. In the close regulation of

[3] Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401. Girard, P., La pression osmotique et le méchanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912.

[4] Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Bartell, F. E., and Madison, O. E., *J. Physical Chem.*, 1920, xxiv, 593.
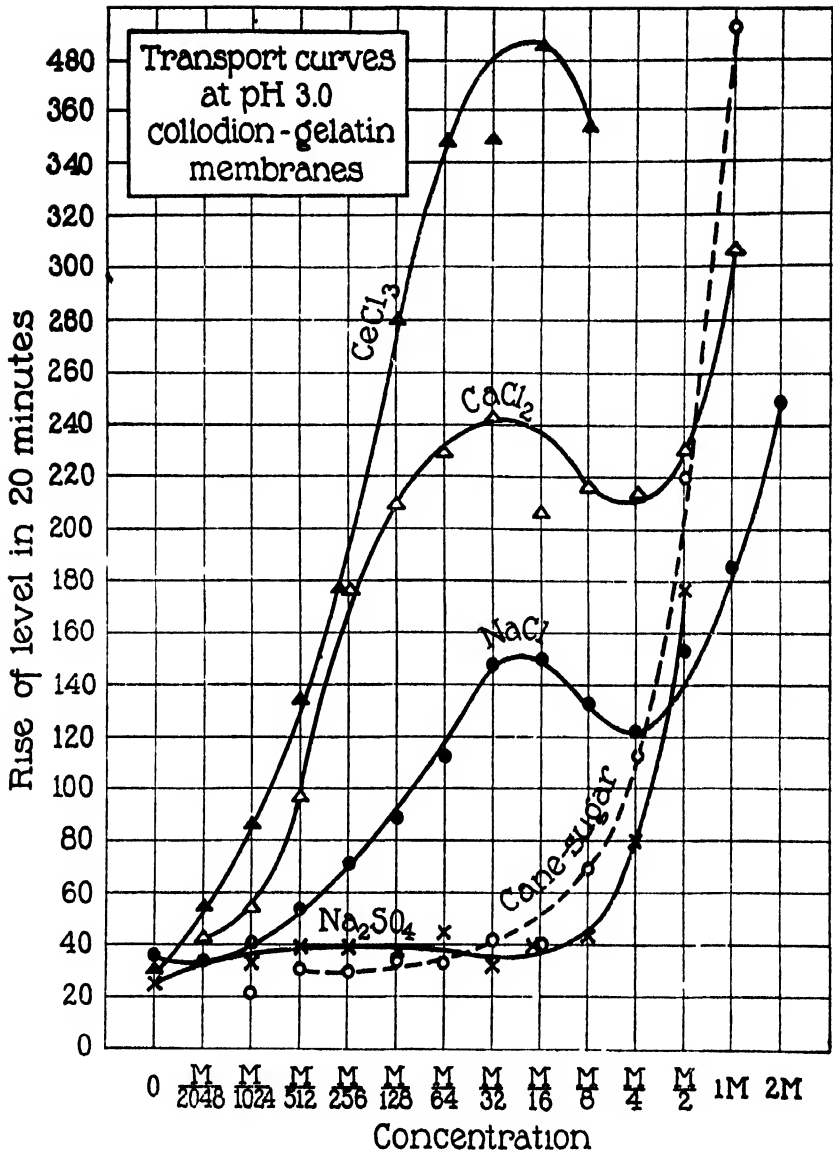
FIG. 1. Transport curves of liquid from the side of water to the side of salt solution through collodion-gelatin membranes of pH 3.0. Abscissæ are the concentration of salt, ordinates the rise in mm. of level in manometer in solution after 20 minutes. Notice the difference between the transport curve for cane-sugar and those for NaCl, CaCl₂, and CeCl₃.

the hydrogen ion concentration the experiments to be given in this paper differ from those of previous workers.

Fig. 1 represents the transport curves for different concentrations of CeCl₃, CaCl₂, NaCl, Na₂SO₄, and cane-sugar, all of pH 3.0 (HCl having been added). The outside solution was an HCl solution also of pH 3.0 (approximately N/1,000 HCl), but containing no salt. The abscissæ are the concentrations, the ordinates the level in mm. to which the liquid had risen in the manometer after 20 minutes. The results of these experiments corroborate similar experiments already published.[5]

The curves for the first three salts, CeCl₃, CaCl₂, and NaCl, rise at first until the concentration is about M/32, then fall and then rise again at a concentration of about M/4. The curves for cane-sugar and Na₂SO₄ commence to rise at a concentration of about M/32 or M/8 respectively. The transport of water therefore increases with the valency of the cation and inversely with the valency of the anion and the question arises how to account for these curves.

According to Helmholtz's formula modified by Perrin, the transport of liquid through a capillary under the influence of a direct current is

$$v = \frac{q \cdot \epsilon \cdot E \cdot D}{4\pi \cdot \eta \cdot L}$$

where v is the quantity of liquid carried electroosmotically, q the cross section of the capillary, ϵ is the potential difference between the two strata of the double layer inside the capillary, E the external electromotive force (acting at right angles to the electrical double layer in the capillary), D the dielectric constant of the medium, η the coefficient of internal friction, and L the distance of the external electrodes.

In the experiments on anomalous osmosis the driving force, E, is not furnished by an external P.D. but by a P.D. across the membrane, which has its origin in the difference between the solutions on the opposite sides of the membrane and which acts only through the extremely small distance of the thickness of the membrane. ϵ is the P.D. between the liquid inside the pores and the wall of the gelatin film. At pH 3.0, gelatin exists in the form of gelatin-acid salts, e.g. gelatin

[5] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 387, 563, 577, 659, 673.

chloride, when the acid is HCl. The water inside the pores of a film of gelatin chloride is negatively charged while the gelatin is positively charged. This is a consequence of the theory of Donnan's membrane equilibrium.[1]

In these experiments the gelatin with which the membrane was coated was originally isoelectric, but the film of gelatin was brought into equilibrium with water of pH 3.0 by putting the collodion bags for several hours into HCl of pH 3.0 before the beginning of the experiment. Since the addition of salts influences both the P.D. across the membrane (*i.e.* the value of E) as well as the P.D. between the liquid inside the pores and the wall of the pore (*i e.* the value of ϵ) it is necessary to measure the influence of salts on these two P.D. separately and then try to use the results for the analysis of the curves in Fig 1. The measurements of the P D. across the membrane were made with the aid of a Compton electrometer and the two electrodes used were calomel electrodes with saturated KCl solution. Measurements of the P D. across the membrane were made at the beginning of the experiment (Table I) and at the end; *i.e.*, after 20 minutes (Table II). The figures for the P D. in Table II are lower than in Table I for the reason that during the experiment part of the salt diffused into the outside solution so that the concentration of the salt solution inside the bag diminished while that in the outside increased; hence the value of E diminished. In addition the hydrogen ion concentration which was the same inside and outside the salt solution at the beginning changed and this added a complication which has been discussed in a previous paper.[6] Table I shows that the P.D. across the membrane (E) increases with the valency of the cation and inversely with the valency of the anion, E being a maximum for $CeCl_3$, being lower for $CaCl_2$, and still lower for NaCl, for Na_2SO_4 it becomes about zero or even slightly negative (Table II). It is, therefore, obvious that in a semiquantitative way the results of Tables I and II (*i.e.* the values of E) explain the difference in the ascending branches of the curves in Fig. 1 up to a concentration of about M/32.

It can be shown that this P.D. is at least partly due to diffusion potentials. In measuring the diffusion potentials the principle of a

[6] Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 213.

TABLE I.

Influence of Concentration of Salt on the Value of E.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl), *at beginning of experiment.* Sign of charge of solution always positive unless minus sign is added.

Concentration.	0	m/2048	m/1024	m/512	m/256	m/128	m/64	m/32	m/16	m/8	m/4	m/2	1 m	2 m
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
CeCl₃	1.0	9.0	15.0	21.0	32.0	41.0	45.0	41.0	41.0	41.5	42.5	47.0	49.0	
CaCl₂	0.0	8.0	16.0	16.0	23.0	25.0	35.0	34.0	33.0	39.0	24.0	25.0	28.0	28.0
NaCl	1.5	5.5	8.0	12.0	13.0	18.0	22.0	20.0	23.0	23.0	-12.0	-17.5		
Na₂SO₄	0.5	5.0	6.0	5.0	8.5	7.5	5.0	-1.5	-2.0	-8.0	-0.5	1.0	0	
Cane-sugar			2.0	2.0	0	0	0	-1.0	0	0				

TABLE II.

Influence of Concentration of Salt on the Value of E.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl), *after 20 minutes from beginning of experiment.* Sign of charge of solution always positive unless minus sign is added.

Concentration.	0	m/2048	m/1024	m/512	m/256	m/128	m/64	m/32	m/16	m/8	m/4	m/2	1 m	2 m
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
CeCl₃	-0.5	10.0	16.0	22.5	28.0	35.0	41.0	33.0	32.0	29.0	24.0	23.0	20.0	
CaCl₂	-1.0	6.5	11.5	17.0	22.0	23.0	30.0	27.5	25.0	25.0	11.0	12.0	10.5	8.0
NaCl	1.5	4.0	7.5	12.0	10.0	13.0	16.5	16.0	16.0	15.0	-10.0	-13.0		
Na₂SO₄	0	3.5	1.0	1.0	1.0	-0.5	-2.5	-6.0	-6.0	-10.0	3.0	5.0	1.0	
Cane-sugar			3.0	6.0	2.0	2.0	3.0	4.0	0	1.0				

flowing junction of Lamb and Larson[7] was used in a simplified and also less accurate form, which, however, gave results of sufficient accuracy for our purpose. Table III gives some of the results showing that the order of efficiency of the various salts and the influence of concentration are the same in diffusion potentials as in the P.D. across the membrane. The diffusion potentials are, however, considerably lower than the P.D. across the membrane (Tables I and II). The diffusion potentials depend on the difference in the relative velocity of the oppositely charged ions of a salt. A comparison of the values in Table I and Table III suggests that the source of the P.D. across the membrane is the same as that of the diffusion potential if we assume that at pH 3.0 the cations experience a greater retardation in the diffusion through protein films than anions.

The figures in Table I, II, or III do, however, not explain the drop in the curves of Fig. 1 which occurs when the concentration of the salt reaches M/32. The cause for this depression lies probably in the influence of the concentration of the salt on the value of ϵ; *i.e.*, P.D. between gelatin chloride and water inside the pores of the membrane. It has been shown in previous experiments that salts depress the P.D. between gelatin particles and surrounding liquid and that the reason for this depression is furnished by the Donnan theory of membrane equilibria.[1] The method of these experiments was briefly as follows:

1 gm. of fine particles of powdered gelatin rendered first isoelectric and of an equal size of grain was put into 200 cc. of various concentrations of a salt (NaCl, $CaCl_2$, etc.) in water and containing 8 cc. of 0.1 N HCl per 100 cc. The gelatin remained in this solution for 2 hours at 20°C. under frequent stirring. The suspension was then put on a filter and the gelatin freed from the supernatant liquid. The gelatin was then melted by heating to 45° and cooled rapidly to cause solidification and the P.D. between the gelatin and the supernatant liquid was then measured with the aid of a Compton electrometer. Table IV gives the result. The reader will notice that in no case do any of the salts cause a rise in the P.D. between gelatin and liquid. The observed P.D. could, however, be calculated with a fair degree of accuracy from Donnan's equilibrium equation.

[7] Lamb, A. B., and Larson, A. T., *J. Am. Chem. Soc.*, 1920, xlii, 229.

TABLE III.

Diffusion potentials of different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl). Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	0	m/2048	m/1024	m/512	m/256	m/128	m/64	m/32	m/16	m/8	m/4	m/2	1 M
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
CeCl₃..........		2.0	4.0	7.0	10.0	16.0	19.0	24.0	31.0	36.0	35.0	43.0	47.0
CaCl₂..........		2.0	3.0	6.0	10.0	13.0	17.0	22.0	26.0	30.0			
NaCl..........		0	0.5	1.5	3.0	5.0	7.0	9.0	12.0	15.0	15.0	17.0	18.0
Na₂SO₄..........		-1.0	-1.0	-1.5	-2.0	-3.0	-5.0	-7.0	-9.0	-12.0	-15.0	-22.0	-3.0
Cane-sugar..........	0	0	0	0	0	0	-0.5	-0.5	-0.5	-1.0	-1.5	-3.0	

TABLE IV.

Influence of Different Concentrations of Salts on the Value of ϵ.

P.D. in millivolts between solid gelatin particles and HCl solution. pH of gelatin particles about 2.8 (in absence of salt).

Concentration.	0	m/8192	m/4096	m/2048	m/1024	m/512	m/256	m/128	m/64	m/32	m/16	m/8
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
NaCl..........	25.5	23.0	25.0	25.0	23.0	21.5	18.5	14.0	10.5	7.0	5.5	2.5
CaCl₂..........	25.5	25.5	23.0	24.0	21.0	19.0	15.5	11.5	7.5	5.0	3.0	2.5
BaCl₂..........	26.0	25.0	24.0	23.0	22.0	18.5	15.0	11.0	7.5	5.5	2.5	2.0
CeCl₃..........	26.0	25.0	22.0	21.5	19.0	16.0	11.5	8.0	5.0	2.5	2.5	
Na₂SO₄..........	25.0	22.0	22.5	21.0	18.0	16.0	11.5	8.5	6.5	4.0	3.0	1.5

The effect of the salt on ϵ was depressing and no increase in ϵ is noticeable in the low concentrations of CeCl$_3$ or CaCl$_2$. It is therefore obvious that the effect of the salts on ϵ exhibited by our method of experimenting can account for the drop in the curves in Fig. 1 but apparently not for the initial rise or the augmenting effect of low concentrations of CeCl$_3$ or CaCl$_2$. If Helmholtz's formula holds for these experiments curves representing the product of the values $E \times \epsilon$ should therefore show an initial rise, followed by a drop; moreover, they should show the relative order of the rise as exhibited in the transport curves in Fig. 1.

In Fig. 2 the curves for $E \times \epsilon$ are plotted with the concentration of the salt as abscissæ and the value of $E \times \epsilon$ as ordinates. The values of ϵ are taken from Table IV and those for E from Table II. The general order of the four curves in Fig. 2 is sufficiently similar to that in Fig. 1 to indicate that our interpretation of the curves in Fig. 1 is approximately correct. Thus the transport curve for Na$_2$SO$_4$ in Fig. 1 is flat, and so is the $E \times \epsilon$ curve for Na$_2$SO$_4$ in Fig. 2. The curves for NaCl, CaCl$_2$, and CeCl$_3$ rise in both figures in the order named and in both curves the rise is followed by a drop. The second rise of the curves in Fig. 1 after a concentration of M/4 is due to the osmotic effect and has no connection with the electrical effect, as is shown by the fact that this rise occurs also in the cane-sugar curve. Hence, as far as the electrical effect in Fig. 1 is concerned, the character of the curves resembles that of the curves in Fig. 2, as is proven by the fact that if we superpose the curves for $E \times \epsilon$ in Fig. 2 over the transport curve for cane-sugar in Fig. 1 we get the curves of the type of Fig. 1. This is still more approximately the case if we substitute for the purely osmotic transport effect not the curve for cane-sugar but the curves for NaCl and CaCl$_2$ at the isoelectric point of gelatin (Fig. 4). Since the curve for NaCl at pH 4.7 (Fig. 4) rises more slowly than the corresponding curve for CaCl$_2$, the lowest point in the NaCl curve in Fig. 1 must be lower than that for CaCl$_2$.

This explains the empirical rule at which the writer had arrived in his previous papers[5] on anomalous osmosis; namely, first, that the transport of water through the collodion-gelatin membrane from the side of the water to the side of solution is increased by that ion of the salt which has the same sign of charge as the membrane, and dimin-
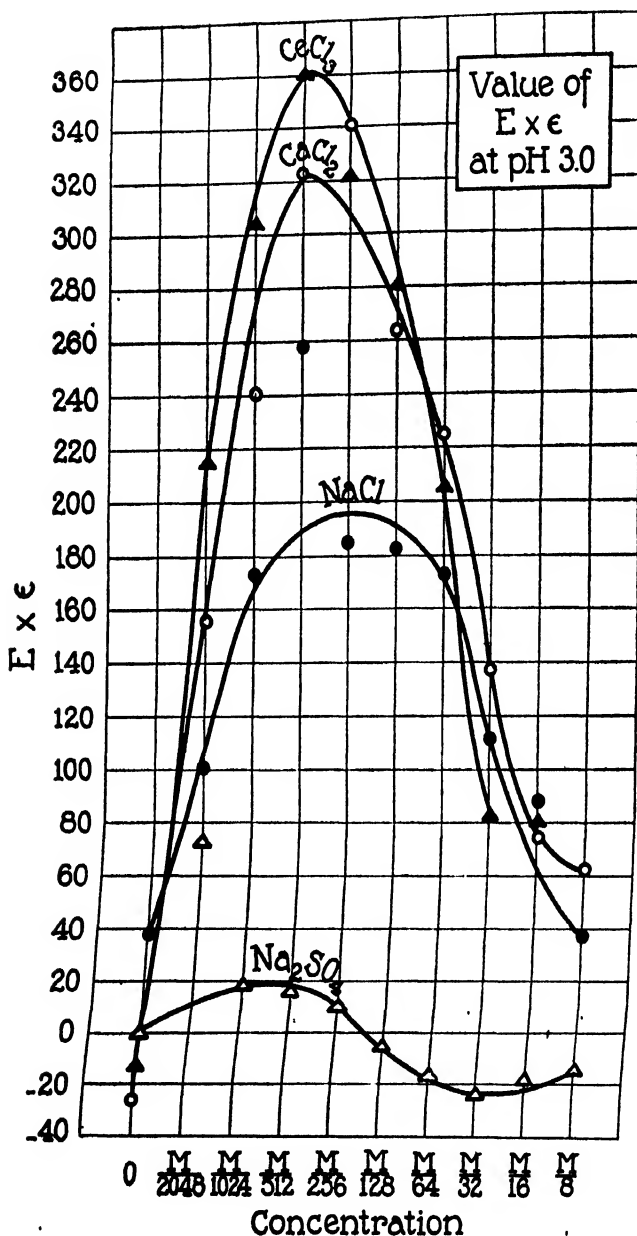
FIG. 2. Value of product of P.D. across the membrane (E) and P.D. inside the pores of the membrane (ϵ) at pH 3.0. Abscissæ are the concentration of salt solution, ordinates $E \times \epsilon$. Notice similarity of curves for $E \times \epsilon$ with the curves in Fig. 1 at lower concentrations of salt.

ished by the oppositely charged ion with a force increasing with the valency of the ion; and second, that the relative effect of the oppositely charged ions is not the same at different concentrations, but that at low concentrations the augmenting effect of the ion with the same sign of charge as the membrane increases more rapidly than the depressing effect of the oppositely charged ion, while at high concentrations the reverse is true. The augmenting effect of the ion with the same sign of charge as that of the membrane on the transport of liquid is due chiefly, if not exclusively, to the effect of the salt on the P.D. across the membrane (E), which depends upon that ion which gives the salt solution the opposite charge from that of the liquid inside the pores. This ion is in this case the cation and hence the transport increases with the valency of the cation (Tables I and II). The depressing effect of the oppositely charged ion on the transport of liquid is due to the effect on ϵ. If ϵ is determined by the Donnan equilibrium between the solid gelatin salt and the bounding solution, it must, according to the theory, be depressed by that ion which has the opposite sign of charge as the protein ion.[1] The oppositely charged ions of a salt act, therefore, each on a different type of P.D., the one on diffusion potentials, the other on a P.D. due to an equilibrium condition.

There are three discrepancies between the curves in Figs. 1 and 2 which need further explanation. First, the location of the maximum of the curves in Figs. 1 and 2 is not identical, being located at M/32 in Fig. 1 and at M/256 or M/512 in Fig. 2. This may be partly or entirely due to the fact that the concentration of the liquid was lower inside the pores than in the salt solution since water was flowing constantly from the side of water into the solution, thus causing a considerable dilution inside the pores.

Second, the curves in Fig. 1 do not come down to zero while those in Fig. 2 come down to nearly zero at a nominal concentration of M/8. For this we may have two reasons, first, that when the concentration exceeds M/4 the transport due to osmotic forces becomes so great that a drop of the transport curves to zero is no longer possible; or it may mean that after the concentration exceeds M/4 a new source of electrification of the gelatin inside the pores not accounted for by the ionization of the protein commences. We shall return to this

possibility later and show that there is no adequate support for this second assumption, though it cannot be absolutely excluded.

Third, the difference between the curves for CeCl₃ and CaCl₂ is smaller in Fig. 2 than in Fig. 1. It is possible that Ce increases the value of ϵ beyond that accounted for by the ionization of gelatin chloride.

Aside from these discrepancies we can say that Helmholtz's formula explains the curves for anomalous osmosis given in Fig. 1 when the values for ϵ used are those to be expected on the basis of the Donnan equilibrium. It may, therefore, be stated that the Donnan theory is able to explain the phenomena of anomalous osmosis more completely than any other theory thus far offered.

II. The Transport Curves on the Alkaline Side of the Isoelectric Point.

In these experiments the salt solutions were rendered alkaline by adding enough KOH to bring the salt solution to a pH of 11.0. The outside solution was a pure KOH solution also of pH 11.0 but free from salt. Fig. 3 gives the curves for the transport of liquid in the solution during the first 20 minutes. The curves show a rise—until the concentration of the salt is about M/64—followed by a drop, and then a second rise follows at a concentration of about M/8. The general character of the curves in Fig. 3 is about the same as that in Fig. 1 but the relative efficiency of the cations and anions is reversed. In solutions whose pH is on the alkaline side of the isolectric point of gelatin, the "attraction" of the solution for water increases with the valency of the anion but inversely with the valency of the cation; while on the acid side the relative efficiency of the two oppositely charged ions is the reverse. Thus in Fig. 1 the curve for Na₂SO₄ is flat while that for CaCl₂ rises; in Fig. 3 the curve for Na₂SO₄ rises while that for CaCl₂ is flat. The reason for this reversal is the fact that the sign of charge between the liquid inside the pores of the gelatin film and the gelatin wall of the pore is reversed on the opposite sides of the isoelectric point. At pH 3.0 the gelatin is positively charged and the liquid inside the pores is negatively charged; while at pH 11.0 the gelatin is negatively charged and the liquid inside the pores is positively charged. The sign of charge of the solution in the P.D. across the membrane, *i.e.* of E, remains, however, the same in alkali and acid solutions (Table V).

Table V gives the P.D. across the membrane at the beginning of the experiments represented in Fig. 3 and Table VI gives the diffusion potentials between the same salt solutions of pH 11.0 against KOH of pH 11.0 without salts and with no membrane between them.
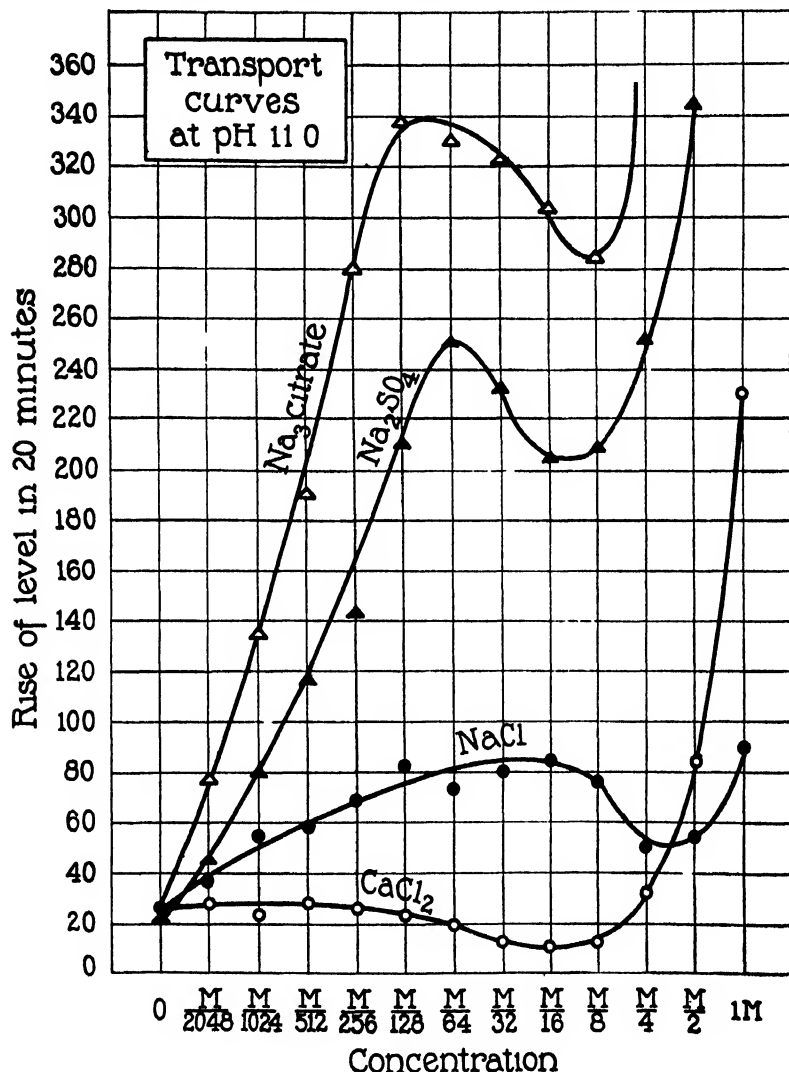
FIG. 3. Transport curves at pH 11.0. Notice reversal of relative efficiency of anions and cations between the curves in Figs. 1 and 3, due to the fact that the sign of charge of liquid inside the pores is positive at pH 3.0 and negative at pH 11.0.

TABLE V.

P.D. across a collodion-gelatin membrane between different concentrations of salts of pH 11.0 against H₂O of pH 11.0 (alkali used, KOH) *at beginning* of diffusion.   Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
Na₃ citrate........	−20.0	−27.0	−38.0	−34.0	−37.0	−39.0	−33.5	−37.0	−44.0	−46.0	−52.0	−56.0
Na₂SO₄........	−15.0	−23.0	−25.0	−28.0	−29.0	−25.0	−23.0	−21.0	−23.0	−26.5	−30.0	
NaCl........	− 5.0	− 8.0	−13.0	− 7.0	−10.0	1.0	4.5	9.5	13.5	19.0	22.0	24.0
CaCl₂........	− 4.0	− 2.5	0	6.5	14.0	20.0	26.5	33.0	38.0	42.5	49.0	50.0

TABLE VI.

Diffusion potentials in millivolts of different concentrations of salts of pH 11.0 against H₂O of pH 11.0 (alkali used, KOH.) Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
Na₃ citrate........	0	−4.0	−6.0	− 9.0	−12.0	−15.5	−20.0	−24.0	−28.5	−33.5	−38.0	−43.0
Na₂SO₄........	0	−2.5	−3.0	− 4.0	− 5.0	− 6.5	− 8.5	−11.0	−14.0	−17.0	−19.5	−22.0
NaCl........	0	1.0	1.5	3.0	5.0	8.0	10.0	12.5	15.0	18.0	20.5	23.5
CaCl₂........	0	4.5	7.0	10.5	15.0	19.5	23.5	28.5	34.0	39.0	43.0	48.0

We can say that the sign and order of the values of the potentials are the same in Tables V and VI indicating that the P.D. across the membrane is essentially of the nature of a diffusion potential. The negative P.D. across the membrane in Table V is, however, always greater than the corresponding P.D. across the membrane in Table VI.

The drop in the curves in Fig. 3 beyond a concentration of M/64 is due again to the diminution of ϵ through the increase in the concentration of salts.

III. The Transport Curves at the Isoelectric Point.

The main purpose of this paper is the investigation of the transport curves at the isoelectric point. At this point the gelatin is not ionized and salts cannot cause a charge of the particles unless they alter the pH or cause the formation of complex protein salts which undergo an electrolytic dissociation. This latter seems to occur when salts with trivalent (or tetravalent?) cations or salts with tetravalent anions are added to isoelectric gelatin, since the addition of this kind of salts has a similar effect as the addition of acid or alkali respectively to isoelectric gelatin. No such effect seems, however, noticeable in the case of salts of the type of NaCl, CaCl$_2$, or Na$_2$SO$_4$. These latter salts influence the transport curves at the isoelectric point in approximately the same way as does cane-sugar or grape sugar; i.e., only osmotically. The transport curves for these latter salts show no electrical effect at the isoelectric point but only the osmotic effect (Fig. 4). In these experiments at the isoelectric point special care was taken that the gelatin film of the membrane was at the isoelectric point at the beginning of the experiment; i.e., that the pH was 4.7. The gelatin used for the film formation was isoelectric and in addition the collodion-gelatin bags were kept in water which had been brought to pH 4.7, by adding acetic acid. The salt solutions also were carefully brought to pH 4.7.

Table VII shows that the P.D. across the membrane is very high at pH 4.7.[8] If, therefore, at this pH there exists only a small P.D.

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[8] If we compare the P.D. across the membrane for the NaCl and CaCl$_2$ and CeCl$_3$ solutions with the diffusion potentials (which are not given in this paper), it is seen that the P.D. across the membrane is greater than the diffusion potentials for the same solutions, as if the membrane retarded the motion of the cations. The same was found when the pH was 3.0. It is, therefore, obvious that this cation retention is not caused by the charge of the gelatin.

TABLE VII.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 4.7 (isoelectric point of gelatin) against H$_2$O of pH 4.7, *at beginning of experiment.* Sign of charge of solution always positive unless minus sign is added.

Concentration.	2 M	1 M	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	0
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
KCl............		0	0	0	0	− 0.5	− 1.0	− 1.0	− 2.5	− 3.5	− 1.0	0.5	1.0	
NaCl...........	0	34.0	33.0	28.0	27.0	28.0	25.0	22.0	19.0	15.0	13.0	10.0	9.0	4.0
LiCl...........	59.0	55.0	57.0	51.0	49.0	39.0	36.0	38.0	32.0	26.0	18.0	14.0	13.0	0
MgCl$_3$..........		75.0	72.5	74.0	64.0	62.5	55.0	52.0	48.0	40.5	35.0	25.0	20.0	4.0
CaCl$_2$..........		71.0	70.0	68.0	62.5	54.0	52.0	44.0	45.0	39.0	33.0	31.5	27.0	5.0
BaCl$_3$..........		64.0	61.0	57.0	57.0	52.0	48.0	43.0	33.0	27.5	25.0	23.0	14.0	3.0
MgSO$_4$..........		5.0	9.0	9.5	10.0	9.0	10.0	11.0	10.0	8.0	6.0	4.0	1.0	1.5
Ce(NO$_3$)$_3$........						55.0	54.0	50.0	49.0	44.0	41.0	28.0	17.0	0
Na$_2$SO$_4$........			−32.5	−28.0	−25.0	−20.0	−18.5	−16.0	−14.0	−11.0	− 8.0	− 7.0	− 4.5	4 0
Na$_4$Fe(CN)				−60.0	−50.0	−45.0	−37.5	−35.0	−33.0	−31.0	−30.0	−30.0	−28 0	
Cane-sugar.		−1.5	− 1.0	3.0	2.5	2.5	3.0	3.5	3.0	4.0	2 5	5.0	2.5	3.0

FIG. 4. Transport curves at the isoelectric point of gelatin. The curves for NaCl, CaCl₂, and Na₂SO₄ resemble the transport curve for cane-sugar showing that osmotic forces suffice to explain these curves. The curve for Ce(NO₃)₃ suggests that this salt confers a positive charge to the gelatin.

inside the pores of the membrane a considerable transport of water by electrical forces must occur. If no such transport is noticeable, it means that ϵ is zero (as the Donnan theory demands, since gelatin
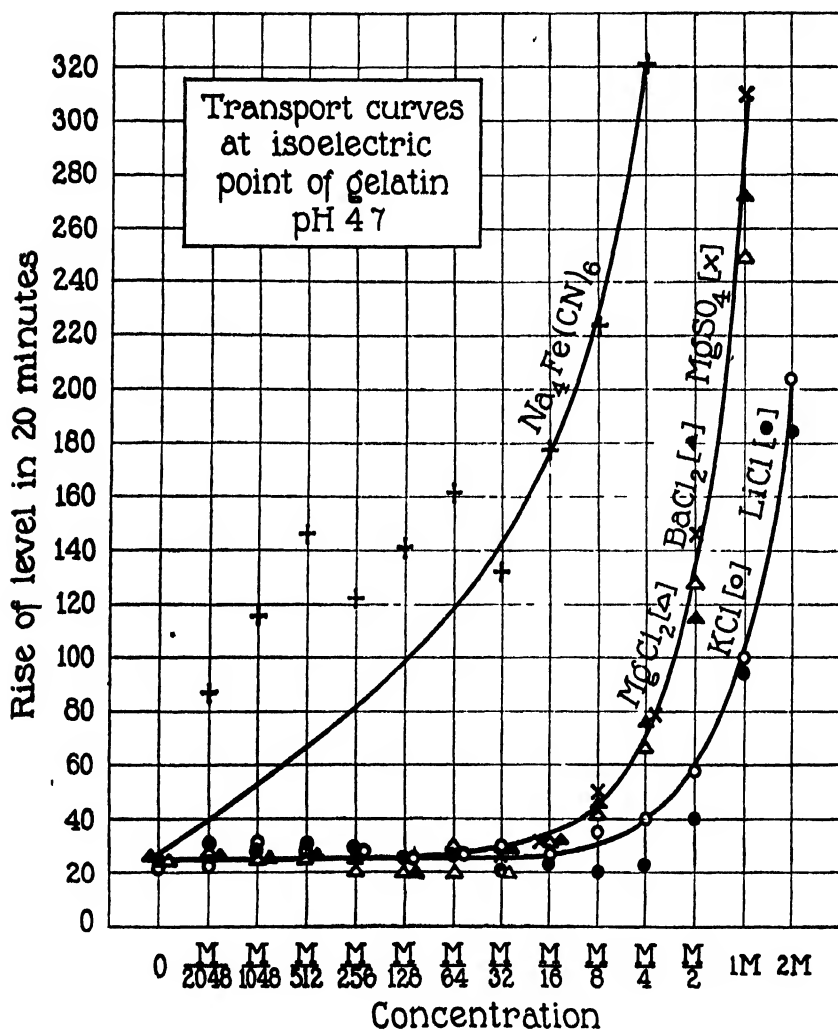
FIG. 5. Transport curves at the isoelectric point. Those for KCl, LiCl, MgCl₂, BaCl₂, and MgSO₄ resemble the transport curves for cane-sugar while Na₄Fe(CN)₆ seems to transfer a negative charge to the isoelectric gelatin.

is not ionized at its isoelectric point and does not combine with salt), and that there is no additional source of P.D. which might be ascribed to adsorption.   Figs. 4 and 5 give the transport curves for cane-sugar, NaCl, KCl, LiCl, $MgCl_2$, $MgSO_4$, $BaCl_2$, $Ce(NO_3)_3$, and $Na_4Fe(CN)_6$ at pH 4.7; *i.e.*, when gelatin is non-ionized.   There cannot be the least doubt that the curves for all these salts (with the exception of $Ce(NO_3)_3$ and $Na_4Fe(CN)_6$) are of the nature of the cane-sugar curve; *i.e.*, they show only that part of the curve which corresponds to the second rise of the transport curves in Figs. 1 and 3, and which must be ascribed chiefly, if not exclusively, to the osmotic forces.   It might be added that the curve for Na_2 oxlate does not commence to rise until the concentration of the salt is M/16.   The initial rise and drop of the transport curves in Figs. 1 and 3, which is the expression of the electrical forces, is entirely lacking in all the curves at the isoelectric point of gelatin, Figs. 4 and 5, with the exception of the curves for $Ce(NO_3)_3$ and $Na_4Fe(CN)_6$, to which we shall now give our attention.

In previous publications the writer has already called attention to the fact that on the alkaline side of the isoelectric point the presence of salts with a trivalent cation has the effect of reversing the sign of the P.D. between gelatin and water.   When the pH is > 4.7, *i.e.* when gelatin exists in the form of Na gelatinate, the Donnan equilibrium causes the expulsion of NaOH from the gelatin into the bounding liquid with the result that gelatin assumes a negative and the bounding liquid a positive charge.   When, however, some $CeCl_3$ or $LaCl_3$ is added the liquid assumes a negative and the gelatin a positive charge.[9]   This reversal of the sign of charge by trivalent cations had been discovered by Perrin in his experiments on electrical endosmose.[10]   The reversal may either be due to a reaction between $Ce(NO_3)_3$ and isoelectric gelatin, in which a compound is formed which dissociates into a complex positively charged gelatin-Ce cation and a negative ion, presumably NO_3, or the addition of the salt brings the pH to a value below 4.7.   In either case the behavior of the curve for $Ce(NO_3)_3$ in Fig. 4 becomes clear.   When $Ce(NO_3)_3$ solutions of pH 4.7 are separated by a collodion-gelatin membrane (of

[9] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 659.

[10] Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50.   Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

pH 4.7) from water of pH 4.7, the $Ce(NO_3)_3$ solution assumes a positive charge as is shown in Table VII. If now the Ce causes the liquid cylinders inside the pores to be charged negatively an electrical transport of water into the solution must occur commencing at a low concentration of the salt and in the way characteristic for the electrical transport curves in Figs. 1 and 3. The curve for $Na_4Fe(CN)_6$ in Fig. 5 suggests that $Na_4Fe(CN)_6$ causes solid isoelectric gelatin to assume a negative charge. The question is, whether this happens only in the case of trivalent (and probably tetravalent) cations and tetravalent anions. It is obvious that all the transport curves for salts with divalent cations, Mg, Ca, and Ba, in Figs. 4 and 5, commence to rise at a slightly lower concentration than the transport curves for the salts with monovalent cation, KCl, NaCl, and LiCl. It might be argued that the salts with bivalent cation transfer also a positive charge to isoelectric gelatin at a concentration above $M/8$ and that the same is true for the salts with monovalent cations, the difference being that the concentration of the salt required for this effect is very high for salts with monovalent cation, slightly lower for salts with bivalent cation, and very low for salts with trivalent cation.

On the other hand it should be pointed out that the transport curve for cane-sugar rises even more rapidly than that for $CaCl_2$ so that there is in reality no need to assume that the $CaCl_2$ charges solid isoelectric gelatin positively. The difference between the transport curves for NaCl and $CaCl_2$ in Fig. 4 is no greater than the difference between the transport curves for cane-sugar and grape sugar. As far as the experiments on anomalous osmosis are concerned, there is no reason to assume that the salts with divalent cations or monovalent cations transfer a positive charge to the isoelectric gelatin or to gelatin of any pH or that the bivalent anions transfer a negative charge, though it is not absolutely disproved that this may not happen at high concentrations of the salts.

It was thus far left undecided whether the positive electrification of isoelectric gelatin by $Ce(NO_3)_3$ in Fig. 4 was due to a change of the pH so that the gelatin was no longer isoelectric or to the formation of a salt between isoelectric gelatin and $Ce(NO_3)_3$ dissociating into a positively charged complex gelatin-Ce ion and negatively charged NO_3 ions. To settle this question experiments were made with

buffer solutions consisting of mixtures of M/50 acetic acid and M/50 Na acetate in the proper proportions to give a pH 4.7, 5.6, or 3.4. Solutions of different concentrations of Ce(NO$_3$)$_3$ were made up in these buffer solutions and put into the collodion bags. The outside solutions
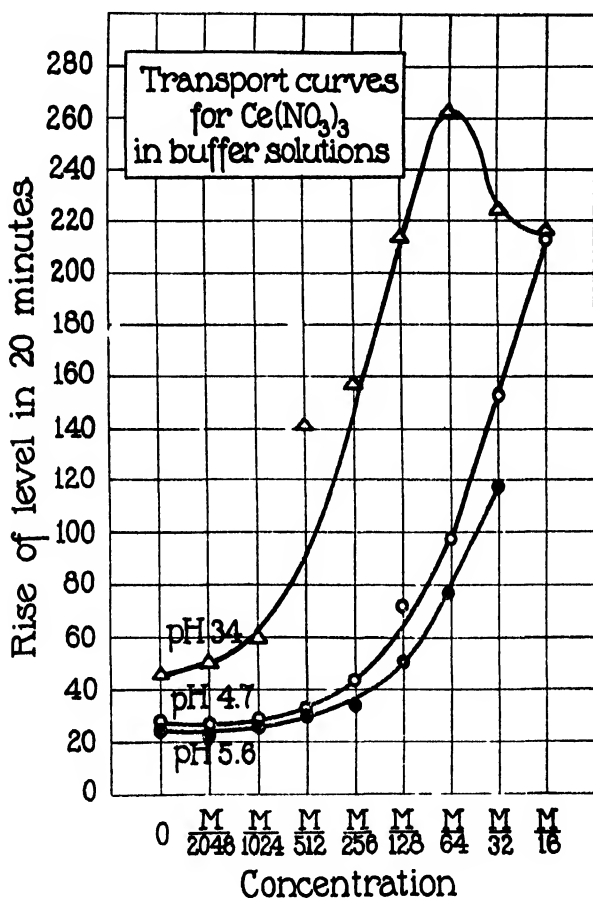
FIG. 6. Influence of Ce(NO$_3$)$_3$ on the transport curve in the presence of buffer solutions (mixtures of M/50 acetic acid and M/50 Na acetate).

were the same buffer solutions without the Ce(NO$_3$)$_3$. The curves in Fig. 6 represent the transport values in 20 minutes. We will first direct our attention to the transport curve at pH 4.7. It is clear that at the isoelectric point the transport curve for Ce(NO$_3$)$_3$ rises again steeply, thus supporting the idea that the Ce(NO$_3$)$_3$ reacts with the

protein in the way suggested, thereby giving rise to a double layer in the pores in which the gelatin is positively and the liquid negatively charged. Table VIII shows that at pH 4.7 there is a high P.D. across the membrane in which the $Ce(NO_3)_3$ solution assumes a positive charge. If now the liquid inside the pores is negatively charged an electrical transport of water from the water side into the solution must occur.

A comparison of the transport curve for $Ce(NO_3)_3$ of pH 4.7 in Fig. 6 with that in Fig. 4 shows that the latter is higher. This finds its explanation in the fact (evident by a comparison of Tables VIII and VII) that the P.D. across the membrane, *i.e.* the value E, is less in the experiment with buffer salt in Fig. 6 than without it in Fig. 4.

TABLE VIII.

P.D. in millivolts across the collodion-gelatin membrane between solutions of $Ce(NO_3)_3$ made up in buffer solution, and buffer solution free from $Ce(NO_3)_3$. The $Ce(NO_3)_3$ solution is always positively charged.

Concentration of Ce(NO₃)₃.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16
pH	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
4.7	0	2.0	4.0	7.0	11.5	16.0	21.0	27.0	34.0
5.6	0	1.0	2.5	4.5	7.5	11.5	17.0	23.0	28.0
3.4	2.0	10.5	17.5	26.0	29.0	32.0	35.0	38.0	44.0

The transport curve at pH 3.4 is higher than at pH 4.7 and the transport curve at pH 5.6 is lower than at pH 4.7 (Fig. 6). Table VIII shows that this finds its explanation in the fact that the P.D. across the membrane varies correspondingly with the pH.

As a consequence it seems possible that the $Ce(NO_3)_3$ causes the positive charge of gelatin by forming a dissociable salt with gelatin in which the positive ion is a complex gelatin-Ce ion. In other words, the salt reacts with isoelectric gelatin in a similar way as acid does. If this assumption is correct the P.D. between gelatin and bounding $Ce(NO_3)_3$ solution must be ascribed to a Donnan equilibrium, in which the $Ce(NO_3)_3$ plays a similar rôle as the HCl.

The difference between gelatin chloride and the hypothetical gelatin-$Ce(NO_3)_3$ salt is this that it is much easier to remove by washing the $Ce(NO_3)_3$ from powdered gelatin than it is to remove the HCl.

In an analogous way we must assume that isoelectric gelatin can combine loosely with $Na_4Fe(CN)_6$ whereby negatively charged complex gelatin-$Fe(CN)_6$ ions and positively charged Na ions are formed.

SUMMARY AND CONCLUSIONS.

1. It has been shown in previous publications that when solutions of different concentrations of salts are separated by collodion-gelatin membranes from water, electrical forces participate in addition to osmotic forces in the transport of water from the side of the water to that of the solution. When the hydrogen ion concentration of the salt solution and of the water on the other side of the membrane is the same and if both are on the acid side of the isoelectric point of gelatin (*e.g.* pH 3.0), the electrical transport of water increases with the valency of the cation and inversely with the valency of the anion of the salt in solution. Moreover, the electrical transport of water increases at first with increasing concentration of the solution until a maximum is reached at a concentration of about $M/32$, when upon further increase of the concentration of the salt solution the transport diminishes until a concentration of about $M/4$ is reached, when a second rise begins, which is exclusively or preeminently the expression of osmotic forces and therefore needs no further discussion.

2. It is shown that the increase in the height of the transport curves with increase in the valency of the cation and inversely with the increase in the valency of the anion is due to the influence of the salt on the P.D. (E) across the membrane, the positive charge of the solution increasing in the same way with the valency of the ions mentioned. This effect on the P.D. increases with increasing concentration of the solution and is partly, if not essentially, the result of diffusion potentials.

3. The drop in the transport curves is, however, due to the influence of the salts on the P.D. (ϵ) between the liquid inside the pores of the gelatin membrane and the gelatin walls of the pores. According to the Donnan equilibrium the liquid inside the pores must be negatively charged at pH 3.0 and this charge is diminished the higher the concentration of the salt. Since the electrical transport is in proportion to the product of $E \times \epsilon$ and since the augmenting action of

the salt on E begins at lower concentrations than the depressing action on ϵ, it follows that the electrical transport of water must at first rise with increasing concentration of the salt and then drop.

4. If the Donnan equilibrium is the sole cause for the P.D. (ϵ) between solid gelatin and watery solution the transport of water through collodion-gelatin membranes from water to salt solution should be determined purely by osmotic forces when water, gelatin, and salt solution have the hydrogen ion concentration of the isoelectric point of gelatin (pH = 4.7). It is shown that this is practically the case when solutions of $LiCl$, $NaCl$, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, Na_2SO_4, $MgSO_4$ are separated by collodion-gelatin membranes from water; that, however, when the salt has a trivalent (or tetravalent?) cation or a tetravalent anion a P.D. between solid isoelectric gelatin and water is produced in which the wall assumes the sign of charge of the polyvalent ion.

5. It is suggested that the salts with trivalent cation, *e.g.* $Ce(NO_3)_3$, form loose compounds with isoelectric gelatin which dissociate electrolytically into positively charged complex gelatin-Ce ions and negatively charged NO_3 ions, and that the salts of $Na_4Fe(CN)_6$ form loose compounds with isoelectric gelatin which dissociate electrolytically into negatively charged complex gelatin-Fe(CN)$_6$ ions and positively charged Na ions. The Donnan equilibrium resulting from this ionization would in that case be the cause of the charge of the membrane.

ELECTRICAL CHARGES OF COLLOIDAL PARTICLES AND ANOMALOUS OSMOSIS.

II. INFLUENCE OF THE RADIUS OF THE ION.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The valency and sign of charge of ions are not the only variables influencing anomalous osmosis. A third variable is the radius of the ion. The radius of the ions of the alkali metals increases in the order of Li < Na < K. The writer has shown that when solutions of pH 3.0 of the chlorides or nitrates of these cations are separated from a solution of water also of pH 3.0 by collodion-gelatin membranes, water diffuses into the salt solution with a rate increasing inversely with the radius of the cation.[1] This is illustrated in the transport curves in Fig. 1 where the abscissæ are the concentration of the salt and the ordinates the number of millimeters to which the level of liquid has risen in 20 minutes in the manometer connected with the solution. It is obvious that the rise is greatest for LiCl, less for NaCl, and still less for KCl. This confirms the results of a preceding publication.

It is also obvious that the three transport curves in Fig. 1 show the initial rise to a maximum at about $M/16$ followed by a drop which is followed by a second rise. This second rise will not interest us here since it is mainly or exclusively the expression of the transport of liquid due to osmotic forces.[2] Only part of the curves, namely, between the concentration from 0 to a concentration of $M/4$, is due to electrical forces, and only these forces interest us in this connection.

The question arises, What determines this influence of the radius of the monatomic and monovalent cations on the electrical transport

[1] Loeb, J., J. Gen. Physiol., 1919–20, ii, 673.

[2] Loeb, J., J. Gen. Physiol., 1919–20, ii, 173; 1921–22, iv, 463.

of water?   The idea prevalent in work on cataphoresis, electrical endosmose, or current potentials seems to be that the charge of the particle or membrane is due to the adsorption of the ions of the salt. Thus the transport curves in Fig. 1 would suggest that the cations
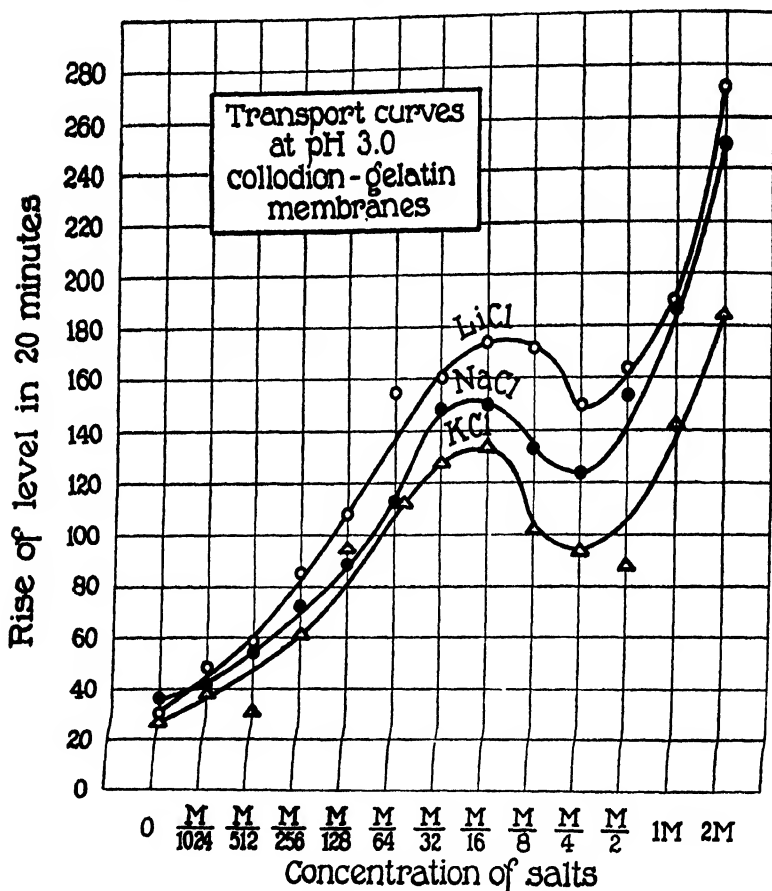
FIG. 1. Influence of Li, Na, and K on rate of electrical transport of liquid through a collodion-gelatin membrane at pH 3.0.   The rate of transport increases inversely with the radius of the cation.

increase the positive charge of the walls of the pores in the gelatin membrane and that this increase occurs inversely with the radius of the three cations.

The influence of the three cations on the charge of gelatin particles was measured directly in the following way.   Doses of 1 gm. of pow-

dered gelatin particles of a definite size (going through a sieve with mesh No. 30 but not through mesh No. 60) were rendered isoelectric and then put into 200 cc. of various concentrations of KCl, LiCl, or NaCl, made up in water containing 16 cc. of 0.1 N HCl at 20°C. After 2 hours, during which the mixtures were stirred frequently, the gelatin was separated from the supernatant liquid by filtration, and after this the gelatin was melted and poured into special glass vessels,
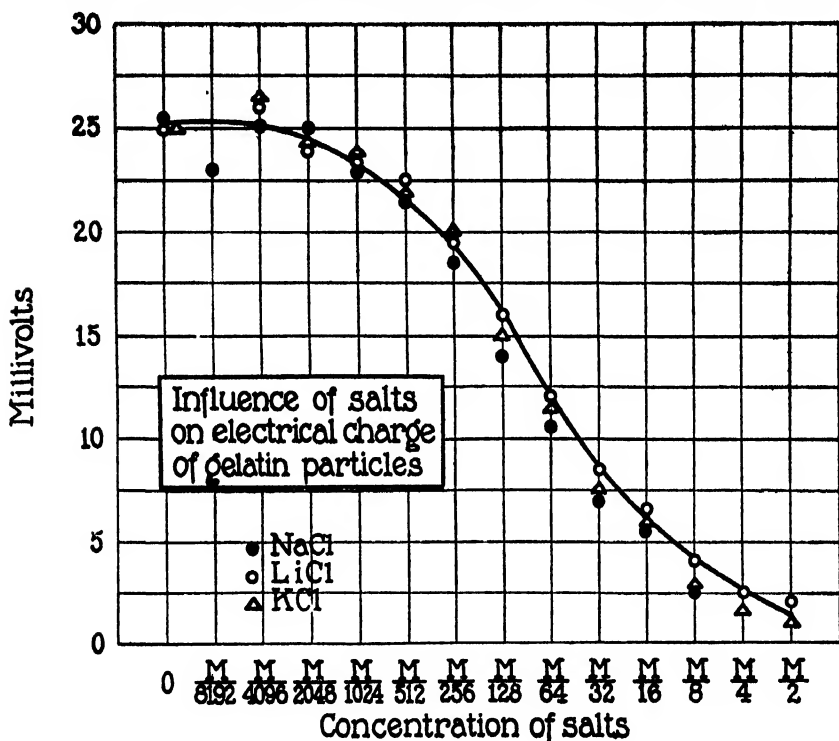
FIG. 2. Influence of LiCl, NaCl, and KCl on P.D. between solid particles of gelatin chloride and surrounding liquid at pH 3.0. This influence is the same for all three salts, suggesting that there exists only a depressing influence of the anion but no opposite influence of the cation on this P.D. Abscissæ are the concentration of the salts; ordinates, observed P.D.

and allowèd to solidify in the vessels standing on ice for 1 hour. The supernatant solution was also cooled in the same way. The P.D. between the solid gelatin and the supernatant liquid with which it was in equilibrium was then measured at a temperature of about 5°C. with calomel electrodes and saturated solutions of KCl by a Compton

electrometer. The details of the procedure can be found in a book which is about to appear.[8]

Fig. 2 shows that the three salts, KCl, NaCl, and LiCl depress the P.D. between solid gelatin chloride and the liquid with which the gelatin is in equilibrium in exactly the same way, since the values expressing the effect of the three salts on the P.D. lie on the same curve (Fig. 2). The ordinates of these curves in Fig. 2 are the observed
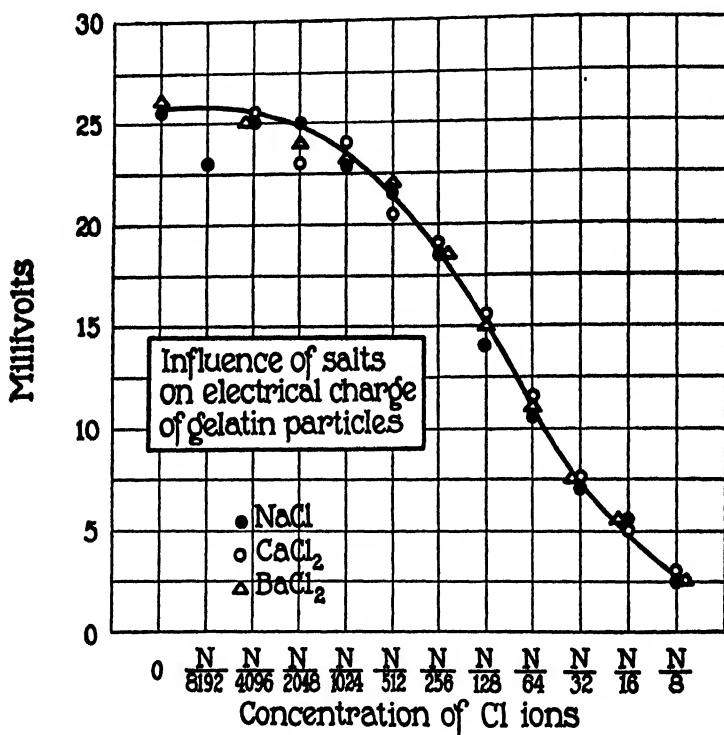
FIG. 3. Influence of NaCl, CaCl₂, and BaCl₂ on P.D. between solid granules of gelatin chloride and surrounding liquid at pH 3.0. Abscissæ are the concentration of Cl; ordinates, observed P.D. The influence is the same for the three salts, proving that there exists only a depressing effect of the Cl ion but no opposite effect of the cation.

P.D. and the abscissæ the concentrations of the salt. These curves contradict the idea that the three cations, Li, Na, and K influence the P.D. between gelatin and water by adsorption, and, moreover, they

[8] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

contradict the idea that the P.D. increases inversely with the radius of the three cations.

It has already been stated in the preceding paper[4] that at pH 3.0 only the anion of the salt has any effect on the P.D between gelatin chloride and the liquid with which it is in contact and that this effect is only depressing. The cation of the salt has no effect. This is shown in Fig. 3, where the influence of NaCl, CaCl₂, and BaCl₂ on the P.D. between solid gelatin chloride and the liquid with which it is in equilibrium are plotted. The method of the experiments was the same as in the experiments with LiCl and NaCl just described. The abscissæ in Fig. 3 are the chlorine ion concentrations (on the assumption of complete ionization) and the ordinates are the P.D. The influence of NaCl, CaCl₂, and BaCl₂ on the P.D. is, therefore, the same for the same concentration of chlorine ions, which means that there exists at pH 3.0 only a depressing effect of the Cl ions on the P.D., but no increase of the P.D. through adsorption of cations. If this latter effect existed the curves in Figs. 2 and 3 representing the influence of salts on the P.D. should not be identical. The identity of the curves in Figs. 2 and 3 can only mean that that ion of a salt which has the same sign of charge as the protein ion has no effect on the P.D. between the particles of gelatin chloride of pH 3.0 and the liquid with which they are in equilibrium. It may be stated incidentally that this was to be expected if the Donnan equilibrium is the cause of the P.D.

If we now return to the interpretation of the influence of the radius of the cation on the transport curves in anomalous osmosis in Fig. 1, we must infer that the difference in the transport curves for KCl, NaCl, and LiCl is not due to any influence of the three cations on the electrical double layer inside the pores of the membrane. We have seen in the preceding paper that there exists a second P.D. which has an influence on the transport curves, namely, the P.D. across the membrane, which is essentially but not exclusively due to a difference in the mobility of the oppositely charged ions. This P.D. was measured at the beginning of each transport experiment and at the end, *i.e.*, after 20 minutes. By that time some of the salt had diffused from the salt solution into the surrounding water. Table I gives the P.D. across the membrane

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[4] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 463.

at the beginning and Table II gives it at the end of the experiment. The salt solution was positively charged and the outside solution was negatively charged. The reader will notice that as soon as the concentration is above M/64 the P.D. across the membrane increases in the order of KCl < NaCl < LiCl, and this corresponds to the difference in the transport curves in Fig. 1 which also begins to become

TABLE I.

Influence of Concentration of Salt on the Value of E.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl), *at beginning of experiment.* Sign of charge of salt solutions positive.

Concentration.	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts
KCl	5.0	5.0	6.5	7.5	8.0	7.5	7.5	5.5	4.5	3.5	3.5	3.0
NaCl	8.0	12.0	13.0	18.0	22.0	20.0	23.0	23.0	24.0	25.0	28.0	28.0
LiCl	6.5	9.0	12.5	18.0	21.0	25.5	27.0	35.0	37.0	37.0	40.0	42.5

TABLE II.

Influence of Concentration of Salt on the Value of E.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl), *after 20 minutes from beginning of experiment.* Sign of charge of salt solutions positive.

'Concentration.	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts
KCl	4.0	4.0	6.0	6.0	6.5	6.0	5.0	3.5	2.5	1.5	1.5	0.5
NaCl	7.5	12.0	10.0	13.0	16.5	16.0	16.0	15.0	11.0	12.0	10.5	.8.0
LiCl	6.5	10.0	13.0	18.0	19.0	24.0	22.0	25.0	22.0	19.5	17 0	15.0

marked when the concentration of the salts rises above M/64. We must, therefore, conclude that the difference in the transport curves in Fig. 1 is due to the difference in the influence of KCl, NaCl, and LiCl on the P.D. across the membrane. This P.D. is, perhaps, essentially, but not exclusively, a diffusion potential. Since K has the greatest and Li the smallest mobility of the three ions, it is to be expected that the diffusion potentials lead to such differences in the

P.D. as are expressed in Tables I and II. This fact has already been discussed, but it may be necessary to return to it in a later publication.

SUMMARY AND CONCLUSIONS.

1. When solutions of KCl, NaCl, or LiCl are separated from water without salt by a collodion-gelatin membrane and when the pH of both salt solution and water are on the acid side of the isoelectric point of gelatin, water diffuses from the side of pure water into the salt solution at a rate increasing inversely with the radius of the cations.

2. The adsorption theory would lead us to assume that this influence of the cations is due to an increase of the P.D. between the liquid and the membrane inside the pores of the gelatin film of the membrane, but direct measurements of this P.D. contradict such an assumption, since they show that the influence of the three salts on this P.D. is identical at pH 3.0.

3. It is found, however, that the P.D. across the membrane is affected in a similar way by the three cations as is the transport of water through the membrane.

4. This P.D. across the membrane varies inversely as the relative mobility of the three cations which suggests that the influence of the three cations on the diffusion of liquid through the membrane is partly if not essentially due to a diffusion potential.

DOES THE KINETICS OF TRYPSIN DIGESTION DEPEND ON THE FORMATION OF A COMPOUND BETWEEN ENZYME AND SUBSTRATE.

By JOHN H. NORTHROP.

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Trypsin, in common with many other enzymes, possesses the peculiarity that the rate of formation of the products of hydrolysis does not increase in proportion[1] to the substrate concentration, as is demanded by the law of mass action, but increases more slowly than the substrate concentration and eventually becomes nearly independent of it.[2] It is necessary to assume, therefore, either that the mass law in its simple form does not hold or that the concentration in grams per liter does not represent the "active" concentration of substrate. A very similar phenomenon is quite common in ordinary catalytic reactions. In this case it has usually been assumed that the mass law is valid but that the concentration to be used in the calculation is not the total concentration of the substance taken but that of some particular molecular species which is formed from this substance in solution. In the case of acid hydrolysis, for instance, the action is assumed to be equal to the concentration, not of the acid itself, but of the hydrogen ions. In this case the assumption is capable of verification since the concentration of hydrogen ions may be determined by several independent methods which give approximately the same results, all verifying the assumption. If this were not the case, the kinetics of acid hydrolysis would be more difficult to interpret than the kinetics of enzyme reactions. In the case of enzymes, however, it has usually been assumed, following the

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[1] Bayliss, W. M., *Arch. Sc. Biol.*, 1904, xi, suppl., 261.

[2] In the case of alkali or acid hydrolysis this is not true. The rate of digestion in the absence of enzyme is proportional to the gelatin concentration. See Northrop, J. H., *J. Gen. Physiol.*, 1921, iii, 715.

suggestions of Henri[3] and of Brown[4] that the law of mass action in its simple form does not hold but that the velocity of the reaction depends upon the decomposition of a compound between the enzyme and substrate. There is a large amount of evidence that a compound is first formed in many chemical reactions and·it has even been stated (Kekule) that no reaction can take place without an addition compound first being formed between the reacting substances. It is quite probable that such a compound is formed in the case of enzyme reactions. The question is whether a sufficient amount of the compound is present at any time to make the kinetics of the reaction depend on the concentration of the compound rather than on the concentration of the reacting substances. Henri,[3] and Michaelis and Menten[5] have attempted to explain the kinetics of invertase hydrolysis by the assumption that the enzyme and substrate combine, according to the law of mass action, to form a compound which subsequently decomposes, liberating the free enzyme and the products of the reaction.[6] It is also assumed that the velocity of hydrolysis depends on the concentration of this compound. It was pointed out by the writer,[7] that if the velocity of hydrolysis depended on the amount of compound formed, then the concentration of substrate required to give the maximum velocity of hydrolysis (*i.e.* to "saturate" the enzyme) should increase with increasing concentrations of enzyme, since it will obviously require more substrate to saturate 100 units of enzyme than it will require to saturate 1 unit. The experiments did not confirm the expectation. It was found that the relative velocity of hydrolysis of different substrate concentrations is always the same, within the experimental error, no matter what enzyme concentration is used (provided the same amount is used with each substrate concentration). It was stated in the article referred to that this was contradictory to the assumption that there was a com-

[3] Henri, V., *Compt. rend. Acad.*, 1902, xxxcv, 916; *Z. physik. Chem.*, 1905, li, 19.

[4] Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

[5] Michaelis, L., and Menten, M., *Biochem. Z.*, 1913, xlix, 333.

[6] It has been shown by Simons in Nelson's laboratory that the method used by Michaelis to measure the initial velocity gives values which cannot be used over the entire course of the reaction.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 595.

pound formed between the enzyme and substrate, since if this were true a greater amount of substrate would be required to saturate a greater amount of enzyme. The experiment, however, is not conclusive, since if it is assumed, as was done by Michaelis and Menten, that the amount of substrate combined with the enzyme is negligibly small, then the difference in concentration of substrate necessary to saturate different amounts of enzyme would be entirely too small to detect experimentally. As far as the relation between the rate of hydrolysis and the concentration of enzyme or substrate is concerned, therefore, the facts may be accounted for by the assumption of an intermediate compound.

It has been shown in a preceding paper[8] that the inhibiting action of the products of the reaction on the trypsin is in quantitative agreement with the assumption that the enzyme and the inhibiting substance combine to form a compound which is inactive and that the rate of hydrolysis is proportional to the concentration of *uncombined* trypsin. It has also been shown that the same assumption will account quantitatively for the protective action of the inhibiting substances when the spontaneous inactivation of the enzyme is followed. The fact that the inhibiting substance protects the enzyme from decomposition is strong evidence that the inhibiting substance combines with the enzyme. In the presence of the substrate, however, the enzyme becomes inactivated at the same rate as the "pure" enzyme[8] (see Ringer).[9] These facts render it unlikely that the enzyme is combined with the substrate. The present paper contains the results of experiments planned to determine whether or not the action of the enzyme with different concentrations of substrate and of inhibiting substances can be accounted for on the assumption of a compound between the enzyme and substrate. The observed facts cannot be accounted for on the basis of the formation of a compound between enzyme and substrate, if it be assumed that this compound is governed by the law of mass action.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 266.
[9] Ringer, W. E., *Z. physiol. Chem.*, 1921, cvi, 107

Experimental Methods.

The rate of hydrolysis was followed by means of the change in conductivity of the solution as already described.[8] The experiments were all conducted at a pH of 6.0.

Trypsin.—The trypsin was a sample of Fairchild's trypsin and was purified for use by dialysis under pressure.

Cooper's gelatin was used and was rendered ash-free by washing at the isoelectric point as described by Loeb.[10] The inhibiting solution was made by allowing trypsin to completely digest gelatin and then concentrating the solution *in vacuo.*

Method of Measuring the Rate of Hydrolysis.—In order to obtain a correct measure of the rate of hydrolysis it is necessary to compare the reactions at the same stage. The rate of digestion decreases rapidly with the progress of digestion for two reasons: first, the concentration of substrate is decreasing; second, the concentration of active enzyme is decreasing owing to the inhibiting action of the products of digestion. If the reactions are compared at a point of equal percentage hydrolysis, the change in substrate concentration is corrected for but the change in enzyme concentration will be very different. The small amount of enzyme will be inhibited to a larger extent than the large amount. If the reactions are compared after equal times, both conditions are varied. If, however, the time to cause a very small amount of hydrolysis is taken, the change in substrate concentration may be considered negligible and the effect on the enzyme will be small and nearly the same in both cases. This method, therefore, gives the most significant value.

The result of an experiment with 1 and 5 per cent gelatin and 1 and 10 units of trypsin is shown in Figs. 1, 2, and 3, in which the increase in specific conductivity of the solution has been plotted against the time in hours. Table I gives the time required to cause an equal percentage of the total change in the two gelatin concentrations with the different enzyme concentrations. The table shows that the time required for the hydrolysis to be completed to any given percentage in the two solutions, is not the same (as would be predicted by the monomolecular formula), but is very much greater for the 5 than for

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[10] Loeb, J , *J. Gen. Physiol.*, 1918–19, i, 237.
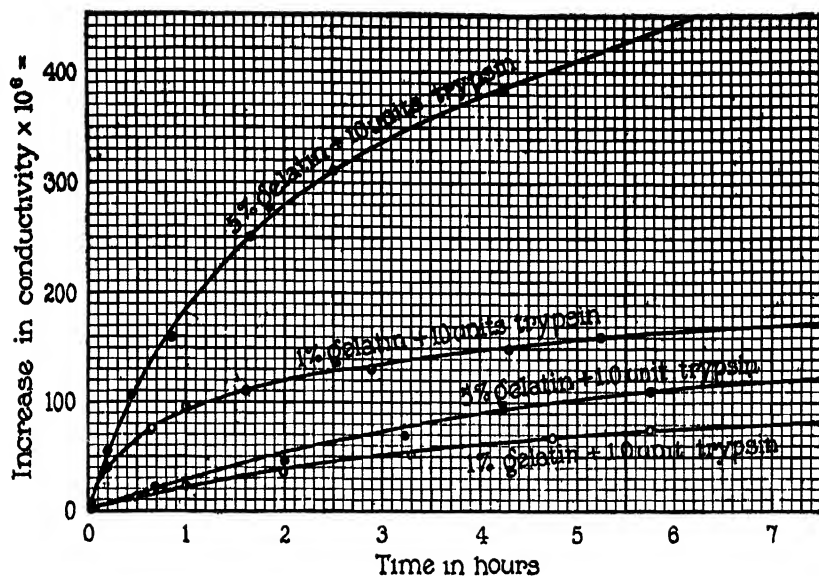
FIG. 1. Rate of digestion of 5 per cent and 1 per cent gelatin with 1 unit and 10 units trypsin as followed by the increase in conductivity.
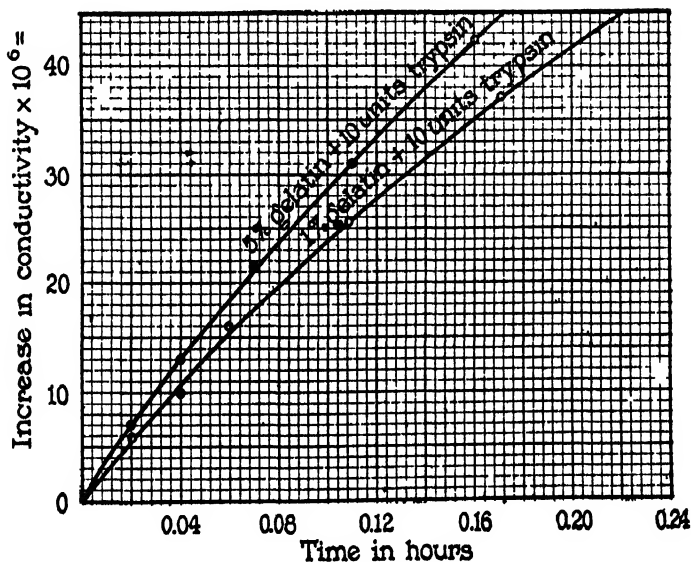
FIG. 2. Large scale of the beginning of Fig. 1. 10 units trypsin.

the 1 per cent gelatin.   The difference is greater with the small amount of trypsin than it is with the larger.   The same result is shown in Table II in which the change in conductivity after an equal time is given.   Here the ratio of the change in the 1 per cent gelatin compared to the change in the 5 per cent gelatin is much smaller when 10 units of trypsin are used than when 1 unit is used.   The results when calculated in this way then seem to show that 1 unit of trypsin becomes "saturated" with gelatin at a lower concentration
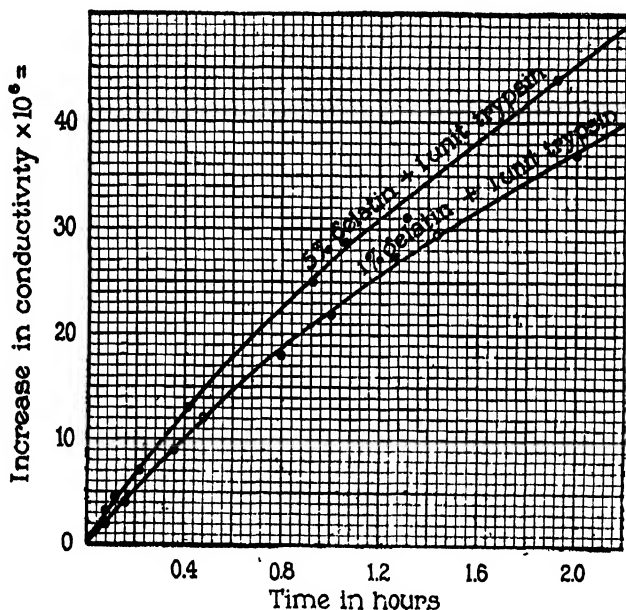
FIG. 3. Large scale of the beginning of Fig. 1.   1 unit trypsin.

of gelatin than do 10 units.   This result, however, is not due to the "saturation" or combination of the enzyme with the gelatin but to the fact that different stages of the reaction are being compared. That this is actually the case is shown in Table III, in which the time required to cause the same amount of hydrolysis is given.   In this case the amount of products formed is the same in both solutions (*i.e.* the stage of the reaction compared is the same) and, as the table shows, the relative velocity of hydrolysis of the 5 per cent gelatin compared to the 1 per cent gelatin is independent of the amount of

trypsin used. The table also shows that this ratio is constant if the first part of the curve is used but later decreases (*i.e.*, the 5 per cent gelatin is hydrolyzed relatively more and more rapidly), as would be expected, since after any appreciable amount of the gelatin is hydro-

TABLE I.

Time Required for Equal Percentage Hydrolysis of 1 and 5 Per Cent Gelatin Solutions When Compared with (a) 1 Unit of Trypsin, and (b) 10 Units of Trypsin.

Complete hydrolysis of 1 per cent gelatin = increase in conductivity of 0.5 × 10^{-3} reciprocal ohms.

Hydroly-sis.	Actual change in con-ductivity.	1 per cent gelatin hydrolyzed with		Actual change in con-ductivity.	5 per cent gelatin hydrolyzed with		Relative rate of hydrolysis of 1 per cent gelatin compared to 5 per cent gelatin.	
		1 unit trypsin. (a)	10 units trypsin. (b)		1 unit trypsin. (c)	10 units trypsin. (d)	1 unit trypsin. $\frac{c}{a}$	10 units trypsin. $\frac{d}{b}$
per cent	*reciprocal ohms* × 10⁶	*hrs.*	*hrs.*	*reciprocal ohms* × 10³	*hrs.*	*hrs.*		
1	5	0.20	0.018	25	0.90	0.085	4.5	4.7
2	10	0.40	0.037	50	2.24	0.17	5.6	4.6
5	25	1.16	0.104	125	7.50	0.57	6.5	5.5

TABLE II.

Change in Conductivity after Equal Time Intervals.

Time elapsed.	1 per cent gelatin +		5 per cent gelatin +		Ratio, change in 1 per cent gelatin change in 5 per cent gelatin tested with	
	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.
hrs.	*reciprocal ohms* × 10⁶	*reciprocal ohms* × 10⁶	*reciprocal ohms* × 10⁶	*reciprocal ohms* × 10⁶		
0.10	2.5	24	3.5	29	0.7	0.8
0.50	12.5	70	15	115	0.8	0.6
1.00	22	95	28	180	0.8	0.5
3.00	52	135	67	335	0.8	0.4

lyzed the two concentrations are no longer as 5:1 but as 5-*a*:1-*a*. As soon as *a* becomes appreciably large compared to 1, the ratio will evidently increase, as *a* increases. The ratio of the time required to cause a given change will therefore decrease. This method may,

therefore, be used to determine the relative rate of digestion of different gelatin concentrations provided the change used as end-point is so small that the gelatin concentration can be assumed to remain constant during the course of the experiment.

TABLE III.

Time Required to Cause an Equal Change in the Conductivity of 1 and 5 Per Cent Gelatin Solutions with 1 Unit of Trypsin and 10 Units of Trypsin.

Increase in conductivity.	1 per cent gelatin +		5 per cent gelatin +		Ratio, time for change in 5 per cent gelatin / time for equal change in 1 per cent gelatin with	
	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.
reciprocal ohms $\times 10^{6}$	*hrs.*	*hrs.*	*hrs.*	*hrs.*		
5	0.19	0.018	0.15	0.014	0.79	0.78
20	0.87	0.08	0.69	0.066	0.79	0.805
50	2.85	0.27	2.05	0.19	0.72	0.71
75	5.75	0.60	3.50	0.30	0.61	0.50

Influence of the Viscosity of the Solution.

It has been suggested that the anomalous results obtained by increasing the concentration of substrate are due to the increased viscosity of the solution. That this is not the cause of the retardation in the present experiments is shown in Table IV which gives the results of an experiment performed with the same gelatin solution which had been kept at 25°C. for varying lengths of time. The viscosity increases slowly under these conditions. As the table shows, the gelatin digests at the same rate whether it has a viscosity of 2.5 times that of water or of 11 times that of water. The physical properties of the gelatin solution evidently have little or no effect on the rate at which it digests.

Influence of the Substrate Concentration.

The results of a series of experiments with varying enzyme and gelatin concentrations are given in Table V. The hydrolysis was followed by the change in conductivity and velocity is taken as the reciprocal of the time required to cause an increase of conductivity

TABLE IV.

Viscosity and Rate of Digestion.

2 per cent gelatin, pH 6.0, specific conductivity 1×10^{-3} (adjusted with NaCl) was heated to 50°C. and cooled rapidly to 25°C. Viscosity was determined at intervals at 25°C. and rate of digestion determined by adding 1 cc. of trypsin to 25 cc. gelatin and following change in conductivity. Increase in formol titration after 1 hr. was also determined.

Viscosity ($H_2O = 1.0$).		Time to change 10 points.	Formol per 5 cc. after 1 hr. (N/50 NaOH).
At beginning.	After digestion.		
		hrs. $\times 10^2$	*cc.*
2.45			
2.90	1.4	70	2.90
		68	2.90
3.8	1.55	70	2.90
3.9	1.5	69	2.95
4.6	1.6	64	2.95
		76	2.97
7.3	1.7	80	2 95
		80(?)	2.90
		62	
11.3	1.8	70	2.95

TABLE V.

Comparison of Observed and Calculated Results with Varying Gelatin and Trypsin Concentrations.

Concentration of gelatin, *S.*	Rate of hydrolysis $\left(= \dfrac{100}{T \text{ hrs. to change 5 points in conductivity}} \right) = C$ with enzyme concentrations (E).					
	$E = 30.0$		$E = 5.0$.		$E = 3.0$.	
	Observed.	Calculated.*	Observed.	Calculated.*	Observed.	Calculated.*
per cent						
6.0	27.3	27.7	4.9	4.6	2.7	2.7
3.0	28.4	25.7	4.6	4.3	2.4	2.6
1.5	25.6	22.5	4.0	3.7	2.2	2.2
0.75	17.5	18.0	3.3	3.0	1.8	1.8
0.38	13.1	12.0	2.4	2.0	1.3	1.3

* Calculated from formula $C = \dfrac{E\,S}{K' + S}$, $K' = 0.5$. The values for E are given at the head of the table.

equivalent to 5 points on the bridge. The table shows that the velocity of hydrolysis increases much more slowly than the substrate concentration and becomes practically independent of it in concentrations of more than 3 per cent.[1] The calculated figures were obtained by assuming that the trypsin and gelatin combined according to the reaction

$$\text{trypsin} + \text{gelatin} \rightleftharpoons \text{trypsin} - \text{gelatin}$$
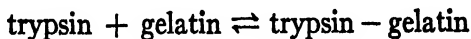
and that the rate of hydrolysis was proportional to the concentration of the *trypsin-gelatin compound.*

Applying the law of mass action to this equilibrium we would have

$$\frac{(E - C) \cdot a\,S}{C} = K\,V$$

or

$$C = \frac{E\,S}{K' + S}$$

in which E equals total amount of trypsin; C, combined trypsin ($=$ combined gelatin); S, amount of gelatin; a, a proportionality factor to change the units of concentration to those of rate of hydrolysis K, the equilibrium constant; V, the volume of solution; and K', a new constant equal to

$$\frac{K\,V}{a}$$

It will be seen that if C is considered negligibly small, compared to E, as well as to S, the equation reduces to Rate $= kC = KES$ which is the ordinary form of the law of mass action.

Evaluation of the Constants.

Since it is assumed that the rate of hydrolysis is proportional to the amount of the gelatin-trypsin compound (C) present, C is the observed velocity.

E, the total amount of trypsin, cannot be determined directly but is taken as a value slightly larger than the maximum value obtained for C, when the substrate concentration is such that the hydrolysis proceeds at the maximum rate. According to the hypothesis, this maximum value is due to the fact that practically all the enzyme is combined, and since C (the rate of hydrolysis) is a measure of the amount combined, E must be very slightly larger. E is, therefore, an arbitrary constant. After a value for E has been determined for a given amount of trypsin solution, the value to be used with any other amount of trypsin will be propor-

tional to the relative amount of trypsin taken, *i.e.*, if E_0 is the value used when 1 cc. of trypsin is taken then nE_0 will be the value for n cc.

K' is determined by substituting the values of C, E, and S and solving for K'. It is, therefore, a second arbitrary constant.

Table V shows that the calculated and observed values are concordant. It also shows that the relative rate of hydrolysis of any two gelatin concentrations with any one trypsin concentration is independent of the value of this trypsin concentration.

The results when the enzyme or substrate concentrations are varied, therefore, agree with those predicted by the assumption that the enzyme and gelatin combine to form a compound and that the rate of hydrolysis is proportional to the concentration of this compound.

The equation which was used to calculate these values, however, contains two arbitrary constants and is of a form to fit any curve which at first shows direct proportionality and then approaches a maximum value. It is not surprising, therefore, that the calculated and observed results agree. The test of an equation of this type is to see whether or not it will fit the results of an experiment other than the one from which the values of the constants was originally obtained. As will be seen below, this is not the case; the equation breaks down when the experiment is performed in the presence of inhibiting substances.

Influence of the Gelatin Concentration on the Retarding Effect of Inhibiting Substances.

It was found[8] in studying the influence of the inhibiting substance on the rate of digestion that the experiments agreed with the assumption that the enzyme and inhibitor combined to form a compound that was inactive and that the rate of hydrolysis was proportional to the concentration of *free enzyme*. It was also found that there was direct evidence that the inhibitor affects the enzyme and not the substrate. The experiment summarized in Table V, however, if taken alone, shows that the influence of the substrate concentration agrees with the assumption that the rate of hydrolysis is proportional to the amount of *enzyme combined* with the substrate. It is evident that both assumptions cannot be correct.

The direct experimental evidence shows conclusively that the enzyme and the inhibiting substances are combined to form a highly dissociated compound, even though the agreement of the experiments with the results calculated from the law of mass action be considered to be accidental. If the substrate is also combined with the enzyme it should be possible, by increasing the substrate concentration sufficiently, to cause all the enzyme to combine with the substrate. In other words, the higher the substrate concentration the less should be the effect of the inhibiting substance. This may be seen from the following equation:

$$\text{trypsin} \quad \overbrace{\begin{array}{l} + \text{ gelatin} \rightleftharpoons \text{trypsin-gelatin} \\ \longrightarrow \\ + \text{ inhibitor} \rightleftharpoons \text{trypsin-inhibitor} \end{array}}$$

Increasing the concentration of gelatin will cause the equilibrium to be shifted in the direction of the large arrow; *i.e.*; it will cause the amount of trypsin combined with the inhibitor to become less. This will be true even though the equilibrium is not one which follows the law of mass action, as long as the equilibrium is reversible and the trypsin-inhibitor compound widely dissociated. It has already been found that the trypsin-inhibitor compound is readily dissociated irrespective of any assumption as to the nature of the compound. Table VI is a summary of experiments made with constant amounts of trypsin and inhibiting substance and increasing concentrations of gelatin. The figures are the averages of four to six determinations. The table shows that the retardation due to the inhibiting substance is independent of the gelatin concentration. In order to account for this result, if the velocity of hydrolysis depends on a trypsin-gelatin compound, it is necessary to assume that the trypsin-inhibitor compound is only very slightly dissociated, and that the inhibiting substance and trypsin are present in about the same concentration. The figures under Calculated I were obtained by means of the law of mass action based on these assumptions. They approximate the experimental values and agree with the experimental result that the percentage retardation is independent of the gelatin concentration. If it be assumed, as was done by Michaelis and Menten, that the inhibiting substance is present in very much higher concentration than the enzyme, the figures given under Calculated II are obtained. They are evidently incompatible with the experiment.

TABLE VI.

Influence of the Gelatin Concentration on the Retardation Caused by the Presence of a Constant Amount of Inhibiting Substances.

Concentration of gelatin, S.	C = rate of hydrolysis in					Ratio of rate of hydrolysis of control inhibitor. Observed.
	Control solution.		Solution containing inhibitor.			
	Observed.	Calculated.*	Observed.	Calculated (I).*	Calculated (II).†	
per cent						
8	10.0	9.5	7.1	6.6	[7.1]	1.43
4	10.0	9.0	7.0	6.3	5.4	1.43
2	9.6	8.1	6.8	5.7	3.7	1.41
1	7.3	6.8	5.0	4.7	2.3	1.46

* Calculated by equation $C = \dfrac{S(E-I)}{K+S}$. $E = 10.1$, $I = 3.0$ (units inhibitor), $K = 0.5$ (derived by assuming that inhibitor-enzyme compound is very little dissociated).

† Calculated by equation $C = \dfrac{E S}{K'' + S} = 10.1$, $K'' = 3.4$, derived by assuming that the amount of inhibitor combined with the enzyme is negligible compared to total amount of inhibitor, and that inhibitor enzyme compound is widely dissociated.

This equation is derived as follows: Let S = total substrate concentration, E total enzyme concentration, C enzyme combined with substrate, J enzyme combined with inhibitor, and I total inhibitor.

Then if the amount of substrate combined with the enzyme is negligibly small compared to the total amount of substrate and if the same is true of the inhibitor, the mass action expressions for the two equilibria are

$$S(E - C - J) = K C \quad (1) \qquad I(E - C - J) = k J \quad (2)$$
$$J = \frac{E S - K C - S C}{S}$$

Substituting this value of J in (2) and simplifying $C = \dfrac{E S}{\dfrac{I K}{k} + K + S}$ in which

K = equilibrium constant of substrate-enzyme equilibrium, and k = equilibrium constant for inhibitor-enzyme compound. Since in this experiment I, K, and k are all constant they may be combined to a new constant K'' and $C = \dfrac{E S}{K'' + S}$.

Effect of Varying the Amount of Trypsin or Inhibiting Substance.

It has been shown above that in order to account for the fact that the percentage retardation is independent of the substrate concentration, it is necessary to assume that the inhibitor-trypsin compound is only slightly dissociated. This assumption, as has already been pointed out, is contradicted by the experiments in which the amount of trypsin or inhibitor is varied. This is shown in Tables VII and VIII. In these tables the results under Calculated I were obtained from the equation used to calculate the results in Table VI, and which is derived by means of the assumption that the inhibitor-trypsin compound is only slightly dissociated. The table shows that the formula will not serve even as a first approximation in spite of the fact that it contains three arbitrary constants. The figures given under Calculated II were obtained by aid of the assumption that the enzyme-inhibitor compound is widely dissociated and that the rate of hydrolysis is proportional to the free enzyme.[11] They agree well with the experimental values.

These experiments show that the results obtained when the gelatin, inhibitor, and trypsin concentrations are all varied cannot be accounted for on the assumption that the trypsin becomes saturated with substrate. They seem to be conclusive even though it is assumed that the equilibria are not governed by the law of mass action, since in order to explain one set of experiments (gelatin constant, trypsin or inhibitor varied) it is necessary to suppose that the trypsin-inhibitor compound is widely dissociated while in the other set of experiments (trypsin and inhibitor constant, gelatin varied) it is necessary to suppose that the same compound is very slightly dissociated. This is true irrespective of the quantitative law that is assumed to govern the equilibrium. There is much more direct experimental evidence in favor of the trypsin-inhibitor compound than of the trypsin-gelatin compound.

There is no doubt on the other hand that the rate of hydrolysis does not increase in proportion to the gelatin concentration as expressed in grams per liter. If it is assumed then that the reaction is

[11] For the derivation of this equation see Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 230.

TABLE VII.

Effect of Increasing the Concentration of Inhibitor.   2 Per Cent Gelatin.

Inhibiting solution.	Rate of hydrolysis = C.		
	Observed.	Calculated (I).*	Calculated (II).†
cc.			
0.0	2.3	2.3	[2.3]
0.125	1.9	[1.9]	1.81
0.25	1.56	1.5	1.45
0.50	1.10	0.72	1.00
1.0	0.65	<0.10	0.58
2.0	0.33	<0.01	0.32

* Calculated from $C = \dfrac{S(E - I \text{ cc.})}{K + S}$; *i.e.*, enzyme-inhibitor compound very slightly dissociated; velocity proportional to *combined enzyme* (C). $E = 2.9$, $S = 2$, $K = 0.5$, $I = 4.0$ units inhibitor per cc. of solution.

† Calculated from $Q = \sqrt{\left(\dfrac{d + K - E}{2}\right)^2 + K E} - \dfrac{d + K - E}{2}$

Assumptions: (1) enzyme-inhibitor compound widely dissociated.   (2) Rate of hydrolysis proportional to *free* enzyme (Q). $K = 2.8$, $E = 2.3$, $d =$ cc. inhibiting solution $\times$ 10.

TABLE VIII.

Effect of Varying Enzyme Concentration with Constant Gelatin and Constant Inhibitor Concentrations.

Gelatin 2 per cent.   25 cc. + 1 cc. inhibiting solution + noted cc. trypsin solution.

Trypsin.	E_1	E_2	Rate of hydrolysis.		
			Observed.	Calculated.*	Calculated.†
cc.					
1.1	13.0	11.0	7.4	[7.4]	[7.4]
0.55	6.6	5.5	3.2	2.3	3.0
0.30	3.6	3.0	1.4	<0.01	1.35
0.15	1.8	1.5	0.80	<0.01	0.70

* Calculated by formula $C = \dfrac{S(E_1 - I)}{K + S}$, $S = 2$, $I = 3.7$, $K = 0.5$, E_1 as in table. Assumptions: (1) enzyme-inhibitor compound slightly dissociated; (2) velocity proportional to combined enzyme.

† Calculated by same formula as (II), Table VII. $d = 5.0$. E_2 as in table above.

governed by the law of mass action and that the velocity of hydroly-
sis is really proportional to the concentration of free trypsin and pro-
tein, it is necessary to suppose that the reaction is confined to some
particular molecular species present in the protein solution or to
introduce a "catalysis" coefficient to express the ratio of actual con-
centration to "active" concentration as has been done in the case of
hydrogen ion by Schreiner.[12] The work of Loeb, Michaelis, Sören-
sen, Robertson, and others has shown that proteins in solution are
ionized so that it would be natural to suppose that the speed of reac-
tion is proportional to the concentration of protein ions instead of to
the total concentration of protein. It has been found that in the
case of pepsin hydrolysis this accounted for the difficulty both as
regards differences in the concentration of protein and the effect of
the hydrogen ion concentration of the solution. In the present case,
however, the ionic concentration, as measured by the conductivity
of the solution, increases more rapidly than the rate of hydrolysis but
less rapidly than the concentration so that the anomaly is only partly
corrected for.

It has been found by von Euler and Svanberg[13] in the case of in-
vertase that the retardation due to inhibiting substances is independ-
ent of the substrate (sugar) concentration so that in the case of this
enzyme also the evidence is contradictory to the assumption of a
substrate-enzyme compound.

Hydrolysis of Mixtures of Casein and Gelatin.

The rate of hydrolysis of casein solutions increases less rapidly than
the concentration of casein, just as in the case of gelatin. This is
shown in Table IX. It is evident that increasing the concentration
of casein above 4 per cent has little or no effect on the rate of diges-
tion. According to the saturation hypothesis the trypsin must,
therefore, be "saturated" with casein when the latter is at a concen-
tration of 4 per cent or more. It is interesting to consider the
digestion of a mixture of casein and gelatin from the points of view

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[12] Schreiner, E., *Z. anorg. Chem.*, 1921, cxvi, 102.
[13] von Euler, H., and Svanberg, O., *Fermentforschung*, 1921, iv, 142.

of the various possible assumptions.[14] The following possibilities present themselves and may be compared with the experiment shown in Fig. 4 and Table X. The values are the mean of 4 to 6 determinations.

TABLE IX.

Effect of Increasing Casein Concentrations on the Amount of Casein Digested.

pH 8.0. Phosphate buffer. 34°C.

| Casein concentration, *per cent*............... . . | 0.5 | 1.0 | 2.0 | 3 0 | 5.0 |
| Increase of amino nitrogen per cc. solution after 1 hr., *cc*............................. .... | 0 20 | 0 33 | 0.45 | 0.54 | 0.55 |
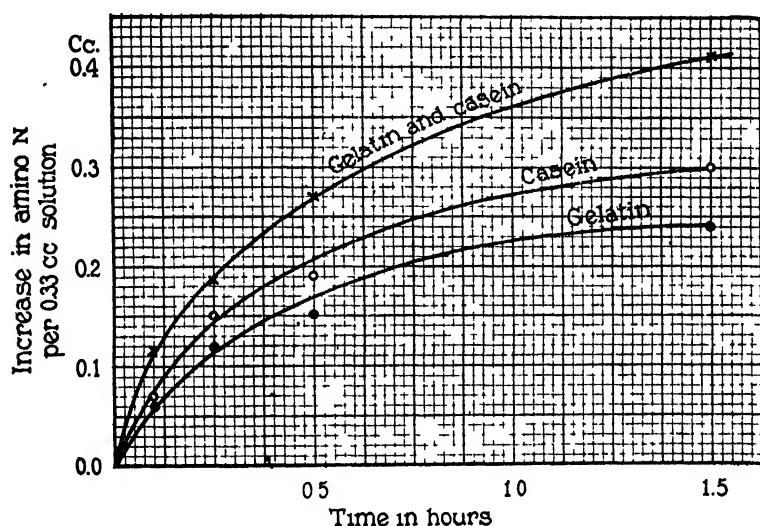
FIG. 4. Digestion curves for 4 per cent casein, 3 per cent gelatin, and a mixture containing both 3 per cent gelatin and 4 per cent casein with the same concentration of trypsin.

[14] This case was considered by Henri and des Bancels (Henri, V., and des Bancels L., *Compt. rend. Soc. biol.*, 1903, lv, 866), who, however, failed to distinguish between the *rate of hydrolysis* and the *amount of hydrolysis*.

TABLE X.

Rate of Hydrolysis of Casein, Gelatin, and a Mixture of Casein and Gelatin.

4 cc. dialyzed trypsin were added to each solution at 34°C. 5 cc. samples were removed after 0.10, 0.25, 0.50, 1.50, and 3.0 hrs. and run into 25 cc. of water containing 10 cc. 0.20 N HCl. 2 cc. of this solution (equivalent to 0.33 cc. of original solution) were analyzed for amino nitrogen by Van Slyke method.

Increase in NH₃ N. (*a*)	Time required to cause an increase of amino nitrogen noted under *a* in		
	Casein solution.	Gelatin solution.	Casein + Gelatin.
cc.	*hrs.*	*hrs.*	*hrs.*
0.1	0.15	0.20	0.09
0.15	0.27	0.40	0.16
0.20	0.48	0.72	0.28

Stage of reaction compared.	Rate of hydrolysis $= \dfrac{1}{T \text{ hrs.}}$ in			
	Casein solution. (*b*)	Gelatin solution. (*c*)	Casein + gelatin.	
			In mixture.	Separately (*b* + *c*).
0–0.10	6.7	5.0	11.1	11.7
0–0.15	3.7	2.5	6.2	6.2
0–0.20	2.1	1.4	3.6	3.5

Casein solution. 4 gm. casein in 100 cc. phosphate buffer. M/10, titrated to pH 7.5.

Gelatin solution 3.5 gm. gelatin in 100 cc. phosphate buffer. pH 7.5.

Gelatin-casein solution. 4 gm. casein + 3.5 gm. gelatin in 100 cc. phosphate buffer as above. pH 7.5.

1. Rate of Hydrolysis Depends on the Concentration of the Enzyme Substrate Compound.

(*a*) The same enzyme acts on both casein and gelatin.

In this case the rate of hydrolysis of the mixture must be less than the rate of hydrolysis of the casein alone, since it has already been assumed, in order to account for the rate-concentration curve, that the enzyme is saturated by 4 per cent casein. The addition of gelatin to the system will therefore remove some enzyme from the casein to combine with the gelatin and since the gelatin hydrolyzes more slowly than the casein, the result will be a decrease in the rate of digestion. This is contradicted by the experiment.

(*b*) The casein and gelatin are hydrolyzed by different enzymes acting entirely independently.

The amount digested at any time in the mixture must then be equal to the sum of the amounts digested at the same time when the casein and gelatin are hydrolyzed separately. The same result would be predicted if the rate of hydrolysis depended on the concentration of free enzymes. This is also contradicted by the experiment.

(*c*) The casein and gelatin are acted on by two different enzymes, but the products formed by either enzyme inhibit the action of the other.

The rate of hydrolysis of the mixture, according to this mechanism, will equal the sum of the rates of hydrolysis of the two separate solutions but the amount of hydrolysis at any given time will be slightly less in the mixture than the sum of the two separate solutions. The same result would be predicted if the rate of hydrolysis were proportional to the concentration of free enzymes.

This is the experimental result.

II. *Rate of Hydrolysis is Proportional to the Concentration of Free Enzyme.*

(*a*) The same enzyme acts on both the casein and gelatin. This assumption predicts that the rate of hydrolysis of the mixture will be equal to the sum of the rates of the two solutions but the amount of hydrolysis of the mixture will be less than the sum of the two separate solutions. This is the experimental result.

We are, therefore, bound to conclude either, first, that the rate of hydrolysis is proportional to the concentration of free enzyme (*i.e.* that the amount combined is negligibly small), or second, that there are two enzymes at work, each of which is inhibited by the products of hydrolysis formed by the other. This latter assumption is gratuitous unless some independent evidence can be found for the existence of two such enzymes. Many experiments were made from this point of view but no evidence could be found for the existence of two enzymes. The ratio of the rate of hydrolysis of gelatin and casein was always the same within the experimental error of about 1 per cent, no matter how the trypsin preparation was treated.

Rate of Hydrolysis as Measured Directly by the Disappearance of the Substrate.

It has been shown above that the rate of formation of the products of hydrolysis of gelatin or casein by trypsin does not increase in proportion to the concentration of substrate but increases much more slowly and becomes independent of the substrate concentration when the latter is more than 2 or 3 per cent. It was also shown that this peculiarity could not be accounted for by assuming the existence of an intermediate compound between the enzyme and substrate nor by the assumption that the hydrolysis was proportional to the ionized protein. In these experiments as in most experiments with enzymes the hydrolysis was followed by determining the amount of the products formed and assuming that the amount of substrate remaining is the difference between the amount of products found at any time and the total amount that can be formed under the most favorable conditions. It is well known that trypsin digestion consists of a series of consecutive reactions since a number of products may be isolated from a digestion mixture which can still be acted on by the enzyme. It seemed possible therefore that the peculiar results discussed above were due to the fact that the increase in the products of reaction does not correctly represent the decrease in the substrate concentration. It is the change in concentration of the latter value that is predicted by the law of mass action. An experiment was therefore performed in which the digestion was followed by determining the increase in amino nitrogen and also the *decrease* in unchanged casein. The results are given in tables XI and XII. The tables show that the two methods give entirely different results. As measured by the increase in amino nitrogen the rate of hydrolysis is practically independent of the casein concentration, whereas when the change in the casein concentration is measured directly the rate of digestion is very nearly proportional to the concentration of casein as demanded by the law of mass action. The constant calculated from the monomolecular formula still shows a drop with increasing hydrolysis. This is more marked in the concentrated than in the dilute solution and is the result expected owing to the inhibiting action of the products of hydrolysis. When the rate of hydrolysis is de-

TABLE XI.

Effect of Increasing Concentration of Casein on the Rate of Hydrolysis as Measured by the Increase in Amino Nitrogen.

Casein dissolved in a mixture of $M/20$ Na_2HPO_4, H_3BO_3, and Na_3 citrate of pH 8.0.   100 cc. of this solution $+$ 1 cc. dialyzed trypsin at $34°C$. for time noted. Amino nitrogen determined by Van Slyke method on 0.6 cc. of solution as noted.

Increase in amino nitrogen per 0.6 cc. solution containing noted concentration of casein.			
After hrs. at 30°C.	Gm. of casein per 100 cc.		
	2 gm.	4 gm.	6 gm.
hrs.	*cc.*	*cc.*	*cc*
0.5	0.20	0.20	0.24
1.0	0.25	0.26	0.25
2.0	0.30	0.38	0.42

The figures are the mean of 4 to 6 determinations.   The average deviation of the mean is about 5 per cent.

TABLE XII.

Effect of Increasing Concentration of Casein on Rate of Digestion as Measured by the Decrease in Undigested Casein.

Casein dissolved in $M/20$ phosphate, borate, citrate buffer pH 8.0.   100 cc· solution $+$ 1 cc. dialyzed trypsin placed at 34°C. and 10 cc. samples removed as noted, titrated to pH 4.6, and added to 100 cc. 0.1 N acetate buffer pH 4.6. Precipitate filtered, dried at 100°C.. and weighed.

		Hrs. at 34°C.			
		0	0.25 hr.	0.50 hr.	1 hr.
Casein per 10 cc. solution, *gm.*	a	0.15	0.08	0.05	0.02
	b	0.31	0.19	0.12	0.08
	c	0.50	0.31	0.26	0.23
Monomolecular constant for solution.	a		1.00	0.96	0.87
	b		0.90	0.84	0.60
	c		0.85	0.60	0.34
Time to dissolve 0.02 gm. in solution, *hrs.*	a	0.08			
	b	0.045			
	c	0.025			
K $=$ concentration casein $\times$ time to dissolve 0.02 gm.	a	0.0120			
	b	0.0140			
	c	0.0125			

termined at the same stage of the reaction by interpolation from the time curves, it is found that the rate of hydrolysis increases in direct proportion to the casein concentration. This is shown by the last line of Table XII. The same result is shown by comparing the monomolecular constants at corresponding values of x. This experiment shows that when the substrate concentration is measured directly the reaction proceeds according to the law of mass action both as regards the concentration of enzyme and of substrate and that the only divergence from the simple monomolecular formula is due to the fact that the enzyme concentration also changes during the course of the reaction, owing to the effect of the products of the reaction, an effect which can easily be demonstrated directly. The experiment described was repeated several times with the same result. It was also found that if the rate of hydrolysis of gelatin was followed by means of the change in viscosity (which is roughly proportional to the gelatin concentration), the same result was obtained. This indicates that in the case of gelatin as well, the apparent discrepancy from the law of mass action is due to the fact that the reaction is really a series of consecutive reactions and that the change in concentration of the original substance cannot be determined from the increase in the total products of reaction. It will be seen that this mechanism will account also for the fact that the increase in the products of reaction as plotted against the time is occasionally a straight line. Assume, for instance, that the reaction may be written $A \rightarrow B \rightarrow C$ and that C is what is determined experimentally. The rate of formation of C will evidently be proportional to the concentration of B at any instant, and this in turn will depend on the relation between its rate of formation and of decomposition. It is possible therefore for the rate of formation of C to remain constant, increase or decrease with time, and there is not necessarily any simple relation between the concentration of A and the rate of formation of C.

SUMMARY.

1. The velocity of hydrolysis of gelatin by trypsin increases more slowly than the gelatin concentration and finally becomes nearly independent of the gelatin concentration. The relative velocity of

hydrolysis of any two substrate concentrations is independent of the quantity of enzyme used to make the comparison.

2. The rate of hydrolysis is independent of the viscosity of the solution.

3. The percentage retardation of the rate of hydrolysis by inhibiting substances, is independent of the substrate concentration.

4. There is experimental evidence that the enzyme and inhibiting substance are combined to form a widely dissociated compound.

5. If the substrate were also combined with the enzyme, an increase in the substrate concentration should affect the equilibrium between the enzyme and the inhibiting substance. This is not the case.

6. The rate of digestion of a mixture of casein and gelatin is equal to the sum of the rates of hydrolysis of the two substances alone, as it should be if the rate is proportional to the concentration of free enzyme. This contradicts the saturation hypothesis.

7. If the reaction is followed by determining directly the change in the substrate concentration, it is found that this change agrees with the law of mass action; *i.e.*, the rate of digestion is proportional to the substrate concentration.

Most of the experimental work in this paper was done by Mr. Frank Johnston.

THE COLLOIDAL BEHAVIOR OF EDESTIN.

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I.

INTRODUCTION.

It has been shown in a series of papers by Loeb[1] that the physical, chemical, and so-called colloidal properties of solutions of the proteins, gelatin, egg albumin, and casein, can be simply explained by two general principles. The first of these is that proteins are amphoteric electrolytes, reacting stoichiometrically with acids and bases to form salts capable of electrolytic dissociation; the second is the principle of Donnan's membrane equilibrium,[2] which is set up when two solutions are separated by a membrane impermeable to one ion of one of the solutions.

The present investigation was undertaken with the object of finding out whether these laws would explain the behavior of solutions of a protein of a different class; namely, a globulin. The globulin selected for the purpose was edestin; and it was found that its solutions obeyed the same laws which had been shown to apply in the case of the other proteins.

The edestin used in these experiments was prepared from ground hemp-seed by the method of Osborne,[3,4] with slight modifications. The hemp-seed meal was extracted three times with 10 per cent sodium chloride solution at 60°C., without previously extracting the oil. The edestin was precipitated by dilution and recrystallized once from sodium chloride solution according to Osborne. The substance was

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922; *J. Gen. Physiol.*, 1918–22, i–iv.

[2] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

[3] Osborne, T. B., *J. Am. Chem. Soc.*, 1902, xxiv, 28, 39.

[4] Osborne, T. B., *Abderhalden's Handb. d. biochem. Arbeitsmethoden*, 1910, ii, 289.

freed from sodium chloride by washing repeatedly with 50 per cent alcohol, and was partly dried by washing with absolute alcohol and then with ether, which left it with a moisture content of 10.1 per cent as determined by heating to constant weight at 110°C. The dry powder was found to contain 18.4 per cent of nitrogen, as determined by a micro-Kjeldahl method accurate to about 1 per cent, whereas Osborne[3] reported 18.69 per cent of nitrogen. Under the microscope the preparation appeared to have the form of fragments of crystals.

The isoelectric point of edestin was given by Rona and Michaelis[5] as at a hydrogen ion concentration of 1.3×10^{-7} (pH 6.89). This was the point of maximum precipitation obtained by using a series of phosphate buffers.[6] Attempts were made to determine the isoelectric point of the present preparation by similar methods, but the point of maximum precipitation seemed to vary with the buffer used. Most of the values obtained, however, lay between pH 5 and 6. Measurements of the osmotic pressure developed in collodion bags by suspensions of edestin in various concentrations of very dilute sodium hydroxide indicated a minimum between pH 5 and 6. Electrophoresis experiments with suspensions of this edestin were made by Dr. John H. Northrop of this laboratory, who also obtained different results with different buffers, which indicated, however, that the isoelectric point appeared to lie between pH. 5.5 and 6.0. The pH of suspensions of this edestin in distilled water was found to be 5.0.

II.

Titration of Edestin with Acids and Bases.

Titration curves of solutions of edestin in acids and bases were obtained in the region where 0.45 gm. could be almost completely dissolved in 100 cc.; i.e., below pH 5.0 and above pH 9.0. Solutions were prepared containing different quantities of acid or alkali but of the same concentration with respect to edestin, and the pH values were

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[5] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193. The edestin used by Rona and Michaelis was apparently a different substance from the present preparation, since they stated that only a small fraction of 1 per cent was soluble in 0.1 N H_3PO_4, while the edestin used in this investigation was completely soluble to the extent of 1 per cent or more in 0.1 N H_3PO_4.

[6] See also Michaelis, L., and Mendelssohn, A., *Biochem. Z.*, 1914, lxv, 1. Using acetate buffers they obtained a value of 2.5×10^{-6} (pH 5.60) for the isoelectric point of a different edestin preparation.

ascertained by means of the hydrogen electrode, using a salt bridge of saturated potassium chloride and a saturated potassium chloride calomel cell. The measurements were made at 33° ± 0.5°, and were referred to 0.1 M HCl as a standard, its pH being taken as 1.036.

The titration curve obtained with 0.45 per cent solutions of edestin in phosphoric acid is given in Fig. 1, Curve I. As a means of determining how much of the acid was combined with the edestin, Curve II was obtained by measuring the pH of mixtures of phosphoric acid and water alone. Curve III, which gives the amount of phosphoric acid apparently combined with the edestin, was obtained by subtracting the ordinates of Curve II from those of Curve I at corresponding pH values. Since phosphoric acid was known to be a weak acid, it was suspected that some of the acid indicated by Curve III to be combined with the edestin might really be present as un-ionized molecules, due to the common ion effect of the ionized edestin phosphate. Accordingly a calculation was made of the first ionization constant of phosphoric acid at 33°, from the data used in plotting Curve II. The results are as follows:

pH	$k = \dfrac{[H^+][H_2PO_4^-]}{[H_3PO_4]}$
2.72	0.0101
2.47	0.0100
2.29	0.0094
2.18	0.0092
1.93	0.0096

These values may be compared with that found by Abbott and Bray at 18°, 0.011.[7] Their constancy indicates that over this range of pH the acid is really monobasic. Accordingly this ionization constant, roughly 0.01, was used to calculate the amount of edestin phosphate actually present, assuming complete ionization of this salt. This was done as follows:

Let c = total concentration of H_3PO_4 (ionized, un-ionized, and present as edestin phosphate),

x = concentration of H_3PO_4 from edestin phosphate (assumed to be completely ionized),

h = concentration of H^+ = concentration of H_3PO_4 from H_3PO_4,

k = primary ionization constant of H_3PO_4,

[7] Abbott, G. A., and Bray, W. C., J. Am. Chem. Soc., 1909, xxxi, 760.
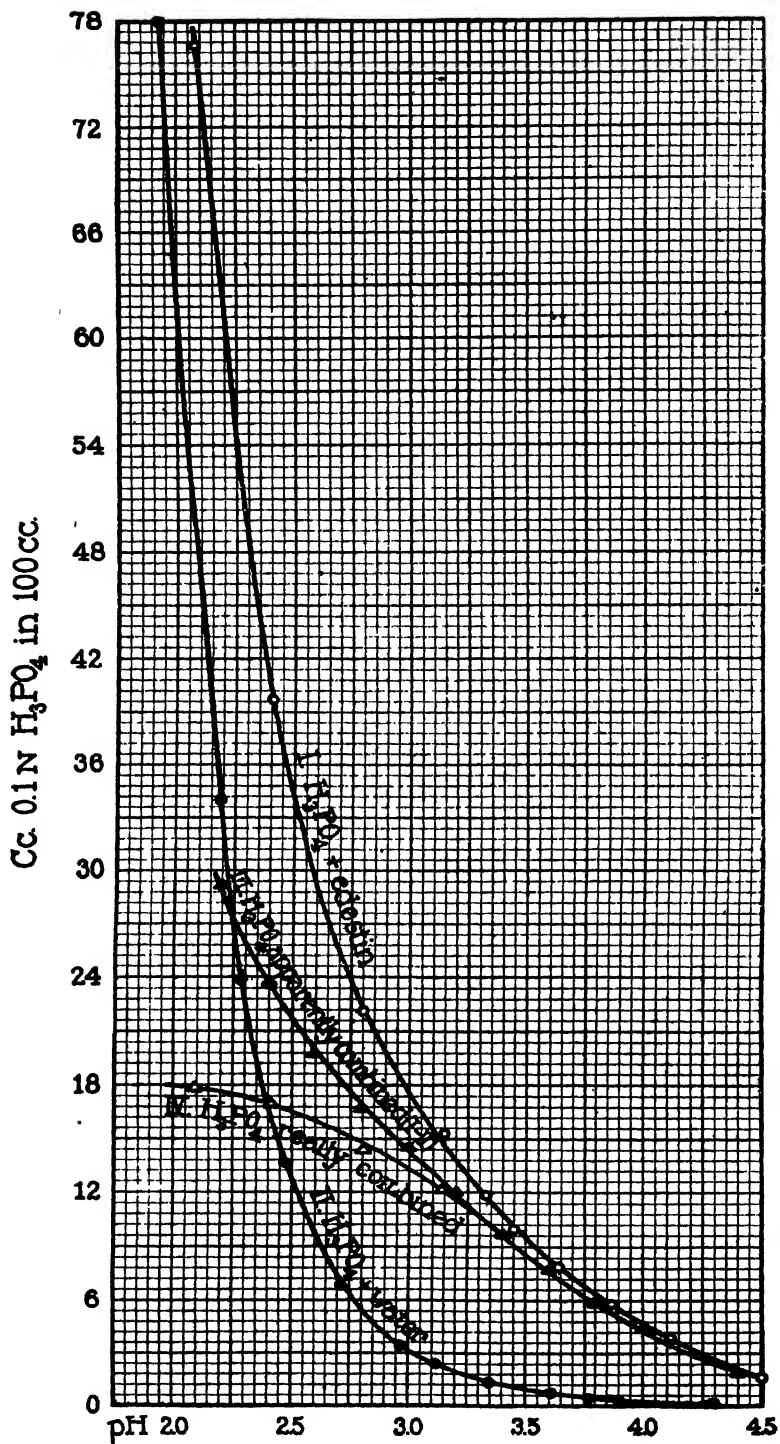
FIG. 1. I. Titration curve of 0.45 per cent edestin with 0.1 N H₃PO₄. II. Titration curve of water with 0.1 N H₃PO₄. III. Difference between I and II. IV. Amounts of H₃PO₄ combined with edestin, calculated from I and ionization constant of H₃PO₄.

Then

$$k = \frac{[\text{H}^+]\,[\text{H}_2\text{PO}_4^-]}{[\text{H}_3\text{PO}_4]} = \frac{h\,(h+x)}{c-h-x}$$

$$= \frac{kc}{h+k} - h$$

The values of x obtained in this way, expressed in cc. of 0.1 N H_3PO_4 per 100 cc., are plotted in Curve IV, which indicates the amount of H_3PO_4 really combined with the edestin. It was found that above pH 3.4 the values so calculated did not differ much from those plotted in Curve III, although it is not strictly justifiable to consider H_3PO_4 as a monobasic acid in this range of pH, since the values obtained for k were not constant above pH 3.

Curves representing the amounts of hydrochloric, sulfuric, and oxalic acids combined with 0.45 gm. of edestin in 100 cc. are given in Fig. 2, along with the curve for H_3PO_4 as given in Fig. 1, Curve IV. In the case of these stronger acids the amount combined was obtained in the same way as Curve III, Fig. 1, by subtracting the ordinates of the acid-water curve from those of the acid-edestin curve. The curves for HCl and H_2SO_4 are nearly identical, indicating that each combines in equivalent proportions with edestin; *i.e.*, H_2SO_4 acts as a dibasic acid. The curve for $\text{H}_2\text{C}_2\text{O}_4$ above pH 4 is identical with with that for H_2SO_4, indicating that here oxalic acid is also dibasic. The curves soon diverge, however, and at the maximum that for oxalic acid is almost exactly twice as high as that for hydrochloric, indicating that here oxalic acid combines not in equivalent but in molecular proportions; *i.e.*, it is here a monobasic acid. The curve for H_3PO_4 seems to reach a maximum at a height a little over three times that of the curve for HCl and H_2SO_4, indicating that H_3PO_4 is combined with the edestin in molecular, not in equivalent proportions, or that H_3PO_4 combines with edestin as a monobasic acid.

In order to show how closely these proportions hold, and in what range of pH, the curves of Fig. 2 were plotted on a large scale and the values given in Table I were read off.

The values in Table I show that the ratios are most nearly as 1:2:3 in the region where the curves become horizontal, indicating that the edestin is all combined.
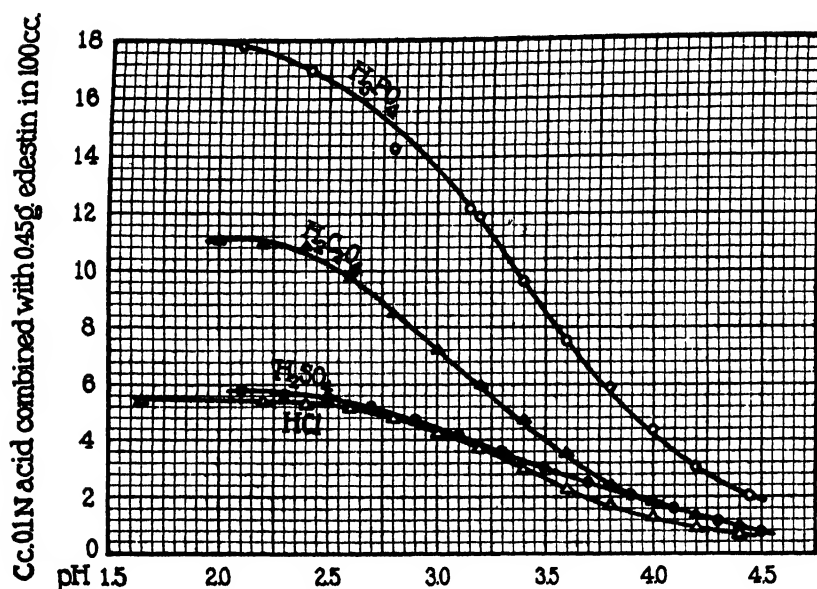
FIG. 2. Amounts of 0.1 N acid combined with 0.45 gm. edestin in 100 cc. Values for HCl, H_2SO_4, and $H_2C_2O_4$ obtained by difference between titration curves with and without protein. Values for H_3PO_4 obtained by calculation, as in Fig. 1, IV.

TABLE I.

Ratios of Amounts of Different Acids Combined with Edestin at Corresponding pH Values.

pH	HCl	H_2SO_4	$H_2C_2O_4$	H_3PO_4	Ratio $\frac{H_2SO_4}{HCl}$	Ratio $\frac{H_2C_2O_4}{HCl}$	Ratio $\frac{H_3PO_4}{HCl}$
	cc.	*cc.*	*cc*	*cc.*			
2.1	5.6	5.8	10.8	17.8	1.0	1.9	3.2
2.4	5.3	5.6	10.8	17.0	1.1	2.0	3.2
2.7	5.05	5.2	9.15	15.4	1.0	1.8	3.0
3.0	4.3	4.4	7.2	13.0	1.0	1.7	3.0
3.3	3.35	3.6	5.35	10.1	1.1	1.6	3.0
3.6	2.3	2.8	3.5	6.95	1.2	1.5	3.0
3.9	1.5	2.05	2.1	4.2	1.3	1.4	2.8
4.2	0.95	1.35	1.3	2.1	1.4	1.4	2.2
4.5	0.6	0.75	0.85	0.8	1.3	1.4	1.3

Attempts were made to carry these curves farther into the region of lower pH, but since they are obtained from differences between the ordinates of steep curves like I and II in Fig. 1, the errors become too

great, as the experimental error in the pH determination is about ± 0.02 pH.
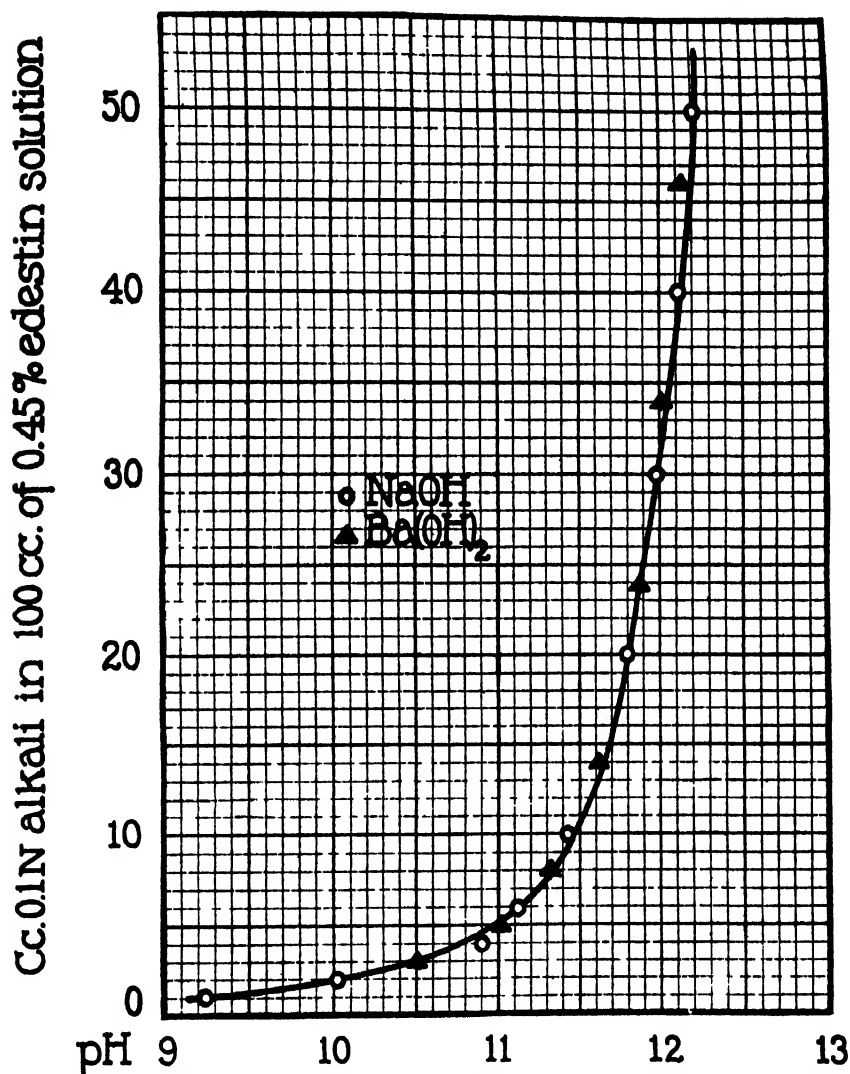
FIG. 3. Titration curve of 0.45 per cent edestin with 0.1 N NaOH and Ba(OH)₂.

Fig. 3 represents the titration of 0.45 per cent edestin solutions with 0.1 N NaOH and Ba(OH)₂. The curve is the original titration curve, and does not represent the amount of alkali combined with the edestin. However, since the points fall on one curve, it seems fairly

evident that the edestin must be combined with these strong alkalies in chemically equivalent proportions.

It should be pointed out that these titration experiments with edestin and acids or bases are completely analogous to those previously obtained by Loeb[1] with gelatin, casein, and egg albumin, and that they are in complete agreement with the idea that proteins are amphoteric electrolytes, reacting chemically and stoichiometrically with acids and bases.

III.

Membrane Potentials.

Loeb[8,9,10] showed that when a solution of gelatin or egg albumin in dilute acid was separated by a collodion membrane from an aqueous solution of the same acid, containing no protein, a difference in electrical potential existed between the two solutions. Moreover, he found it possible to calculate the magnitude of the potential difference with considerable accuracy from the hydrogen ion concentrations of the two solutions, on the basis of Donnan's theory,[2] according to which, for 25°C.,

$$\text{P.D.} = 59 \log \frac{x}{y} \text{ millivolts} \tag{1}$$

where x represents the hydrogen concentration of the outside solution and y that of the inside solution. He showed also that the presence of increasing concentrations of a neutral salt decreased the P.D., the decrease being dependent on the concentration and valence of the anion.

Experiments were carried out to determine whether similar results could be obtained with edestin. Solutions were prepared containing 0.45 gm. of edestin in 100 cc. of HCl of concentration sufficient to give a pH of 3, and containing varying concentrations of Na_2SO_4, NaCl, $CaCl_2$, or $LaCl_3$. These were placed in 50 cc. collodion bags fitted with rubber stoppers and manometer tubes, and the bags were suspended in beakers of HCl of pH 3, which had been made up to

[8] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557.
[9] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.
[10] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 351.

have the same salt concentration as the protein solutions. The beakers were set in a water thermostat at 25° ± 1°C. After equilibrium had been established (12 to 48 hours), the osmotic pressure was measured in terms of millimeters of the solution in the manometer tubes. The P.D. between the inside and outside solutions was determined in each case with the aid of saturated KCl calomel electrodes and a Compton electrometer. The P.D. measurements were made in a room at about 20°C., but the solutions were very nearly at 25°, since the P. D. was determined for each solution within 2 or 3 minutes after removing it from the thermostat at 25°. Finally the pH of the inside and outside solutions, at 33°, was determined with the hydrogen electrode and potentiometer. The calculated P. D. values were reduced to 25° by multiplying by $\frac{298}{306}$ the differences between the E.M.F. readings obtained for the two solutions with the hydrogen electrode. (This amounts to the same thing as using equation (1), but avoids slight arithmetical errors due to rounding off the values for pH.) The P.D. values were read to 0.5 millivolts, but the reproducibility was of the order of 1 millivolt.

The results of experiments with 0.45 per cent edestin chloride, at pH 3, and the four salts mentioned, are given in Tables II to V.

The excellent agreement of the observed and calculated values for the P.D. in these salt experiments proves that the Donnan equilibrium governs the effect of salt on the P.D. of edestin chloride solutions fully as well as in the case of gelatin or albumin chloride. Tables II and III show that here too it is the anion of the salt which has the depressing effect on the P.D., the sulfate ion being more effective than the chloride ion. An increase in the concentration of an ion of opposite charge to that of the protein ion tends to prevent the forcing of acid from the inside to the outside solution, and hence decreases the difference in pH and the resulting P.D. This may also be shown clearly from the results with the different chlorides by plotting the P.D. against the equivalent concentration of chloride ion furnished by the salt, assuming complete or equal ionization of the inorganic chlorides. The results in Tables III, IV, and V are so plotted in Fig. 4. It is evident that the effects of NaCl, CaCl₂, and LaCl₃ are identical, if compared at the same concentration of chloride ion. This proves beyond a doubt that the valence or nature of the cation is of no influence on the P.D. of edestin chloride.

TABLE II.

Effect of Sodium Sulfate on the P.D. *and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of Na$_2$SO$_4$.	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192	M/16,384	0
pH inside..............	Edestin precipitated.	2.93	2.86	2.83	2.88	2.90	2.93	2.97	2.99	3.00
pH outside..............		2.92	2.83	2.78	2.77	2.74	2.72	2.70	2.69	2.69
Observed P.D., *millivolts* .		0.5	2.0	4.0	4.5	9.5	13.5	16.0	17.0	19.0
Calculated P.D., *millivolts* .		0	1.5	3.5	7.0	9.5	12.5	15.5	17.0	18.0
Observed osmotic pressure, *mm*..............			6	10	24	48	71	106	144	172	204

TABLE III.

Effect of Sodium Chloride on the P.D. *and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of NaCl.	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	0
pH inside..............	2.79	2.78	2.79	2.77	2.84	2.84	2.89	2.89	2.95	3.00
pH outside..............	2.79	2.77	2.77	2.74	2.77	2.74	2.75	2.70	2.72	2.70
Observed P.D., *millivolts* ..	0	0.5	1.0	2.0	3.0	6.5	8.0	10.5	14.0	17.5
Calculated P.D., *millivolts* .	0	1.0	1.0	2.0	4.0	6.0	8.0	10.5	13.5	17.5
Observed osmotic pressure, *mm*..............	8	17	27	34	41	56	88	124	152	201

TABLE IV.

Effect of Calcium Chloride on the P.D. *and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of CaCl$_2$.	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	0
pH inside..............	2.82	2.80	2.80	2.88	2.82	2.87	2.92	2.95	2.98	3.02
pH outside..............	2.82	2.78	2.78	2.78	2.75	2.75	2.72	2.70	2.72	2.70
Observed P.D., *millivolts* ..	0	0.5	1.5	3.0	5.0	8.0	11.0	14.0	15.5	15.5
Calculated P.D., *millivolts* .	0	1.0	1.0	6.0	4.0	7.0	11.5	14.5	15.5	18.5
Observed osmotic pressure. *mm*..............	19	26	34	42	55	83	121	151	177	205

TABLE V.

Effect of Lanthanum Chloride on the P.D. *and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of LaCl₃.	M/24	M/48	M/96	M/192	M/384	M/768	M/1,536	M/3,072	M/6,144	0
pH inside...............	2.79	2.79	2.80	2.79	2.80	2.85	2.87	2.95	2.98	3.00
pH outside...............	2.78	2.78	2.77	2.75	2.74	2.74	2.70	2.70	2.70	2.69
Observed P.D., *millivolts* ..	0	0.5	1.5	3.5	4.5	6.5	10.5	13.5	15.5	18.5
Calculated P.D., *millivolts* .	0.5	0.5	2.0	2.5	4.0	7.0	10.0	14.5	16.5	18.5
Observed osmotic pressure, *mm*...............	20	26	33	41	54	82	117	154	180	199

TABLE VI.

Effect of pH on P.D. *and Osmotic Pressure of 1 Per Cent Edestin Chloride.*

pH inside....................	1.47	2.04	2.59	3.06	3.44	3.91	4.38	4.78	5.09	
pH outside....................	1.43	1.91	2.32	2.61	2.90	3.23	3.61	3.91	4.15	
Observed P.D., *millivolts*.........	5.0	7.5	14.0	23.0	29.0	39.0	42.5	37.0	32.5	
Calculated P.D., *millivolts*.......	2.0	8.0	16.0	26.0	32.0	40.0	46.0	52.0	56.0	
Observed osmotic pressure, *mm*..	94	239	415	535	526	501	390	270	200	

TABLE VII.

Effect of pH on P.D. *and Osmotic Pressure of 1 Per Cent Edestin Chloride in* M/512 *Sodium Chloride.*

pH inside....................	1.47	2.02	2.51	2.92	3.30	3.69	4.20	4.73	5.09	
pH outside....................	1.43	1.90	2.29	2.62	2.98	3.38	3.94	4.48	4.81	
Observed P. D., *millivolts*........	2.0	7.5	12.5	17.0	18.0	16.5	12.5	8.0	4.5	
Calculated P. D., *millivolts*.......	2.0	7.5	12.5	17.5	18.5	18.5	15.5	14.5	16.5	
Observed osmotic pressure, *mm*..	82	191	328	383	331	246	134	65	28	

TABLE VIII.

Effect of pH on P.D. *and Osmotic Pressure of 1 Per Cent Edestin Acetate in* M/100 *Sodium Acetate.*

pH inside.....................	3.25	3.45	3.57	3.74	3.94	4.10	4.21	4.39	4.51	4.67
pH outside....................	3.12	3.35	3.48	3.64	3.86	4.04	4.15	4.34	4.46	4.62
Observed P.D., *millivolts*.........	6.5	6.0	5.5	5.0	4.5	4.5	3.5	3.0	2.5	2.5
Calculated P.D., *millivolts*........	7.5	6.0	5.5	6.5	4.5	3.5	3.5	3.0	3.0	2.5
Observed osmotic pressure, *mm*...	139	114	102	88	71	62	56	49	44	38

The effect of the hydrogen ion concentration on the P.D. was determined by using 1 per cent solutions of edestin in varying concentrations of HCl without the addition of salt.   The results of such an experiment are given in Table VI and Fig. 5.
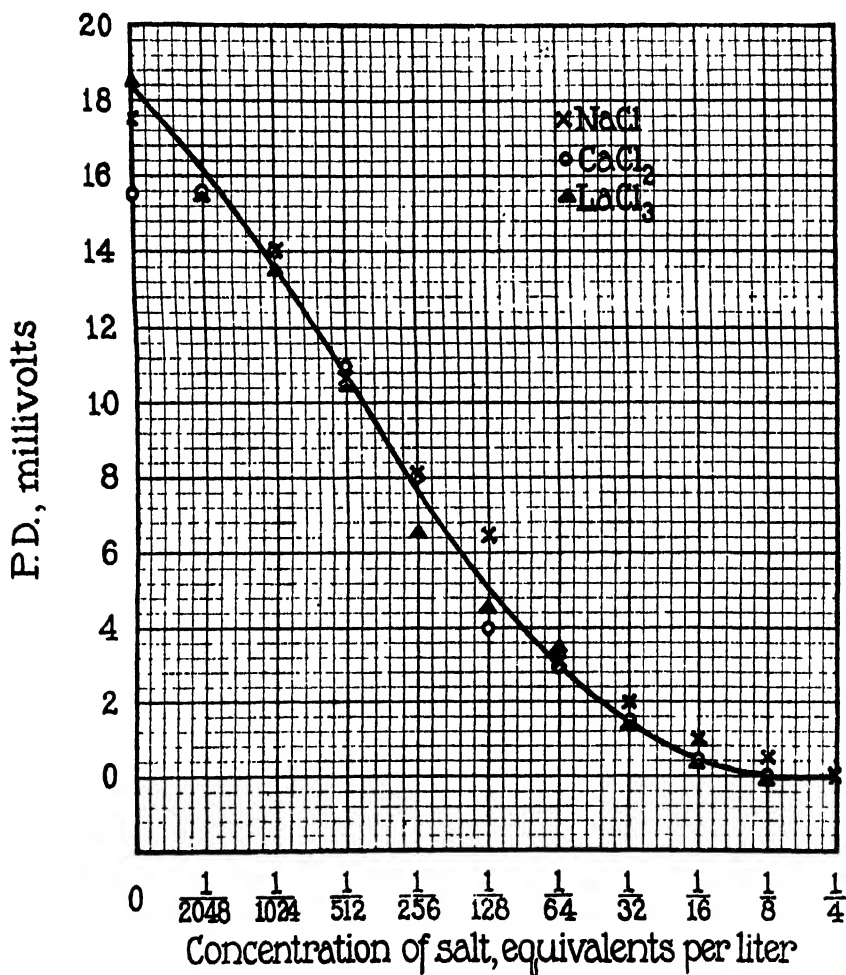
FIG. 4. Effect of different chlorides on P.D. observed with 0.45 per cent edestin chloride at pH 3.

It will be noticed that the observed and calculated values for P.D. agree fairly well up to pH 4.0.   The results therefore show that Donnan's theory does apply quantitatively to edestin chloride solutions between pH 1.5 and 4.0.

Since it had been noticed that the agreement between observed and calculated P.D. was better when the solution had a higher concentration of electrolyte, the experiment of Table VI was repeated with all solutions M/512 with respect to NaCl. The results are given in Table VII.

The agreement with the theory is excellent up to pH 3, but again becomes poor above pH 4. In comparing Tables VI and VII it may be observed that the presence of M/512 NaCl had more effect in decreasing the P.D. of those solutions which contained less HCl. This
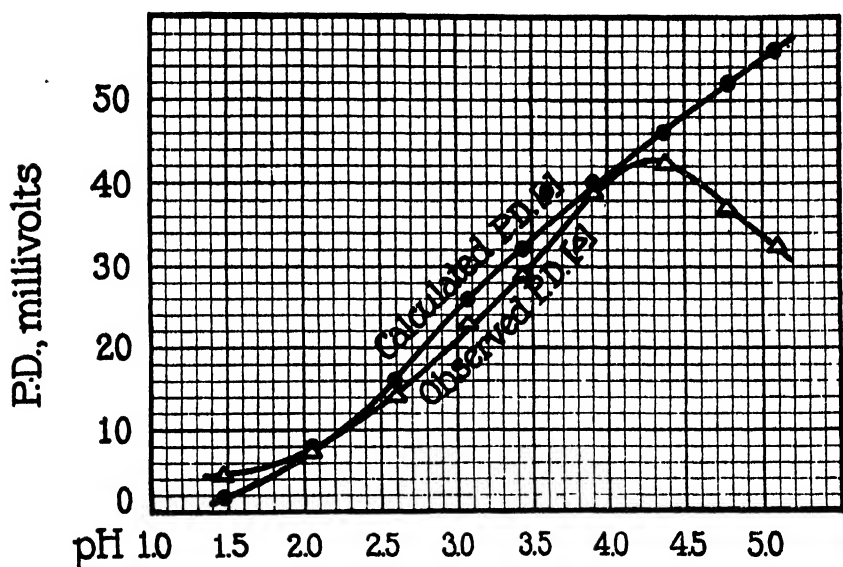
Fig. 5. Effect of pH on P.D. observed and calculated for 1 per cent edestin chloride.

is in line with the experiments already described on the effect of salt concentration on the P.D., indicating that the depression of the P.D. is due to the anion.

In order to obviate any error in the calculated P.D. which might be due to difficulty in determining accurately the pH of the very dilute acid in some of the outside solutions, an experiment was run in which both inside and outside solutions were buffered by M/100 sodium acetate and different concentrations of acetic acid. The results are given in Table VIII and Fig. 6.

It will be observed that the agreement between the observed and calculated values for the P.D. is within the experimental error, and that the experiment includes that range of pH where the values did not agree well in the case of the solutions without buffer. The curve in Fig. 6 is of a different shape from that in Fig. 5 because the depressing action of the anion of the salt has more effect in the less acid solutions, since the concentration of the anion furnished by the acid is originally less in such solutions. The existence of a difference in pH in such buffered solutions and the agreement between the observed
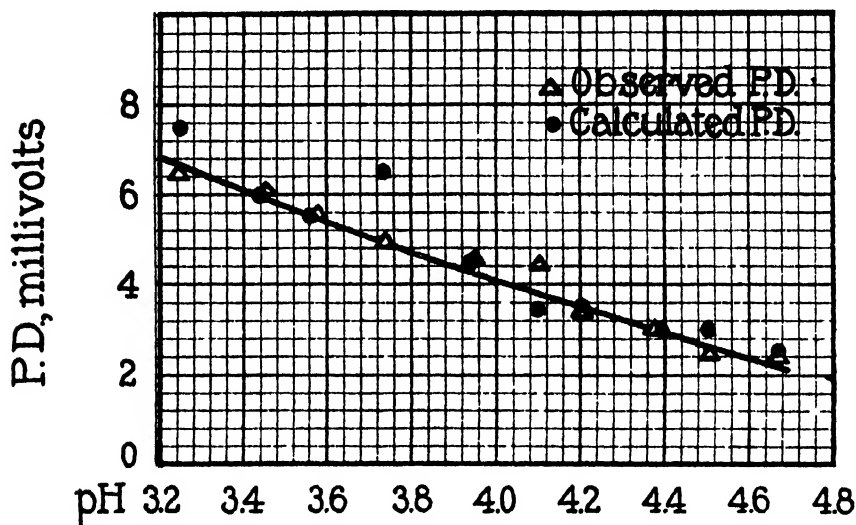
FIG. 6. Effect of pH on P. D. observed and calculated for 1 per cent edestin acetate in M/100 sodium acetate.

and calculated values for the P.D. constitute a striking proof of the validity of Donnan's theory. This experiment, therefore, supplements the results in Tables VI and VII by proving that Donnan's theory applies to solutions of edestin in acid in the region near the isoelectric point as well as in more acid solutions.

A further consequence of Donnan's theory, as applied by Loeb to amphoteric colloids, is that the pH inside should become less than the pH outside on the alkaline side of the isoelectric point of the protein; *i.e.*, the P.D. should be opposite in sign to that found on the acid side. This was tested by working with solutions of edestin in NaOH. It

was found that the solubility of edestin in acid or alkali was less than 0.45 gm. in 100 cc. for a broad zone around the isoelectric point, from about pH 5 to pH 9. Table IX gives the results of a few measurements that were made with 0.45 per cent sodium edestinate in sodium hydroxide. Both inside and outside solutions were protected from the CO_2 of the air by soda-lime tubes.

These results show that the prediction of the theory was confirmed, for the pH outside is now greater than the pH inside, and the sign of both observed and calculated P.D. is opposite to that obtained with the acid solutions. Moreover, the agreement between observed and calculated P.D. is close enough to show that the Donnan theory applies quantitatively on the alkaline side of the isoelectric point of edestin.

TABLE IX.

P.D. and Osmotic Pressure of 0.45 Per Cent Sodium Edestinate.

pH inside..............................	9.93	10.41	10.96
pH outside...........................	10.12	10.55	11.05
Observed P.D., *millivolts*.............	−9.5	−8.5	−6.0
Calculated P.D., *millivolts*...........	−11.0	−8.0	−6.0
Observed osmotic pressure, *mm*.......	28	37	45

IV.

Osmotic Pressure.

The similarity between the depressing effect of salt on the osmotic pressure and on the P.D. in the case of gelatin chloride has been pointed out by Loeb.[9] That the same resemblance exists in the case of edestin chloride is shown by the results in Tables II to V, and by a comparison of Fig. 7 with Fig. 4. Since the abscissæ in Fig. 7 represent equivalent concentrations of salt with respect to chloride ion, and the points obtained with the three chlorides all fall on the same curve, it is evident that it is again only the anion that is effective in depressing the osmotic pressure of a solution in which the protein ion is positive. The nature or valence of the cation seems to have no effect. These results are in contradiction to the theory that the effect of a salt on such colloidal properties of protein solutions is due to the adsorption of both ions of the salt, but are in complete accord with the results of

Loeb on gelatin. The true explanation of the effect of salts on these properties of protein solutions is found in Donnan's theory of membrane equilibrium.
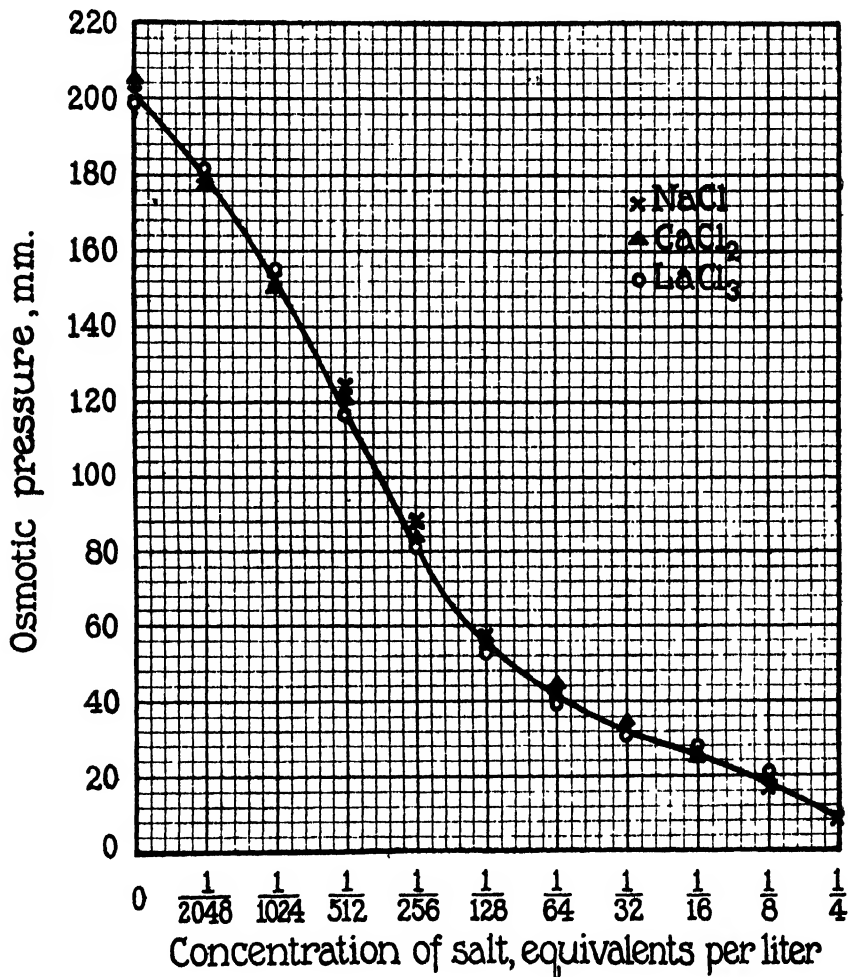
FIG. 7. Effect of different chlorides on osmotic pressure observed with 0.45 per cent edestin chloride at pH 3.

In studying the effect of hydrogen ion concentration on the osmotic pressure of gelatin chloride, Loeb[9] found that the curves obtained were of the same general shape as the P.D. curves, but that the maximum for osmotic pressure was at a slightly lower pH. He later[10]
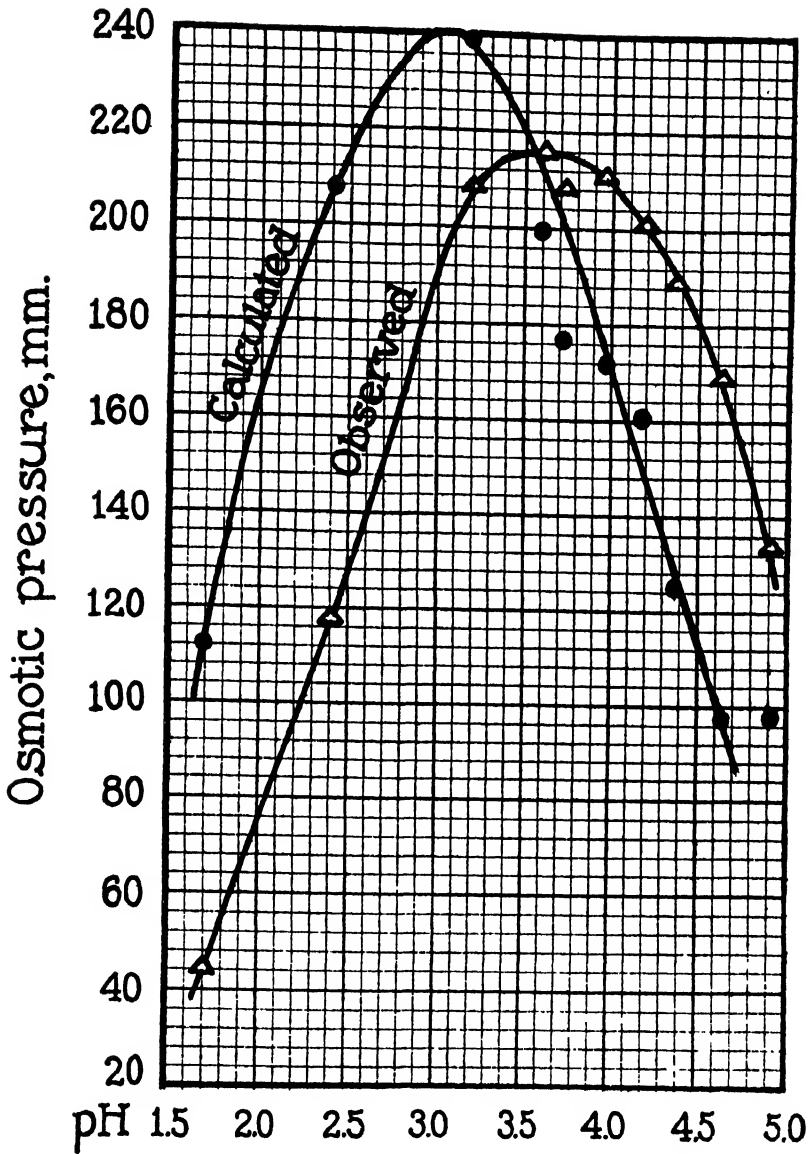
FIG. 8. Effect of pH on observed and calculated osmotic pressure of 0.45 per cent edestin chloride.

showed that the same was true for the chloride of egg albumin.   On
the basis of Donnan's theory he devised a method[11] of calculating the
osmotic pressure of a protein-acid salt solution from measurements
of the hydrogen ion concentration.   The calculation, which neglects
the unknown osmotic pressure of the protein itself, gives the following
expression for the osmotic pressure in the case of a protein-acid salt
with univalent anion:

Osmotic pressure at 24°C. $= 2.5 \times 10^5 (2y + z - 2x)$ mm. of water.

Here y is the hydrogen ion concentration of the inside solution, x that
of the outside solution, and z the concentration of anion from the
protein-acid salt, all being expressed in moles per liter.   Since accord-
ing to Donnan's theory

$$x^2 = y (y + z)$$

the expression for osmotic pressure reduces to

$$2.5 \times 10^5 \times \frac{(x - y)^2}{y}$$

In applying this calculation to gelatin chloride, Loeb found that the
curves representing osmotic pressure as a function of pH were of the
same general shape and height as the observed curves, but had maxima
at a lower pH.   A calculation made from his results on albumin
chloride[10] yielded similar results.

Fig. 8 represents the effect of pH on the observed and calculated
osmotic pressure of a 0.45 per cent solution of edestin chloride.   The
curve for observed osmotic pressure has a maximum at a lower pH than
that for observed P.D. shown in Fig. 5, while the curve for calculated
osmotic pressure has a maximum at a still lower pH.   Thus the
behavior of edestin in these respects exhibits the same peculiarities
which Loeb observed with gelatin and egg albumin.

SUMMARY.

1. It has been shown by titration experiments that the globulin
edestin behaves like an amphoteric electrolyte, reacting stoichio-
metrically with acids and bases.

[11] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 691.

2. The potential difference developed between a solution of edestin chloride or acetate separated by a collodion membrane from an acid solution free from protein was found to be influenced by salt concentration and hydrogen ion concentration in the way predicted by Donnan's theory of membrane equilibrium.

3. The osmotic pressure of such edestin-acid salt solutions was found to be influenced by salt concentration and by hydrogen ion concentration in the same way as is the potential difference.

4. The colloidal behavior of edestin is thus completely analogous to that observed by Loeb with gelatin, casein, and egg albumin, and may be explained by Loeb's theory of colloidal behavior, which is based on the idea that proteins react stoichiometrically as amphoteric electrolytes and on Donnan's theory of membrane equilibrium.

THE ELIMINATION OF DISCREPANCIES BETWEEN OBSERVED AND CALCULATED P.D. OF PROTEIN SOLUTIONS NEAR THE ISOELECTRIC POINT WITH THE AID ' OF BUFFER SOLUTIONS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The writer has shown in a series of papers[1] that the P.D. observed between a solution of gelatin chloride or albumin chloride inside a collodion bag and an outside solution free from gelatin could be calculated from the difference in the hydrogen ion concentration between the inside and outside solutions. The agreement between the observed and calculated values was perfect when the solution contained a neutral salt or when the hydrogen ion concentration of the solution was not too close to that of the isoelectric point; the agreement was, however, less satisfactory when the pH was near that of the isoelectric point of gelatin, *i.e.*, near pH 4.7, and no salts were present.[2] The source of this disagreement seemed to lie in the inaccuracy in the measurement of the pH of the aqueous solution free from gelatin (the outside solution) at a pH between 4.0 and 7.0. If this surmise was correct, the disagreement in that region of hydrogen ion concentrations should be caused to disappear by the use of a buffer solution inside and outside.

1 per cent solutions of isoelectric gelatin were made up in m/100 Na acetate solutions containing varying amounts of 1 m acetic acid so that the pH of the gelatin solution varied (at the end of the experiment) between 4.65 (*i.e.*, practically isoelectric gelatin) and 3.34 (Table I.) ᐧ Collodion bags, of a content of about 50 cc., were filled

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351.
[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 138, 156.

with these solutions of gelatin in buffer solutions as described in previous papers. The bags were put into beakers containing 350 cc. of identical solutions of M/100 Na acetate and 1 M acetic acid as those inside the bags, except that the 350 cc. outside solutions contained no gelatin. The temperature was 24°C. After 24 hours the osmotic pressure, the P.D. between inside and outside solutions, and the pH of the inside and outside solutions were measured. From the value pH inside minus pH outside the P.D. was calculated, and Table I shows that the P.D. thus calculated agrees with the observed P.D. The rest of the table needs no explanation.

TABLE I.

Influence of pH on P.D. *of Solutions of Gelatin Acetate in the Presence of Buffer Solution.*

Cc. 1 M acetic acid in 100 cc. inside and outside solutions.	1.0	1.5	2.0	3 0	4.0	6.0	10.0	15.0	20.0	30.0
Osmotic pressure, in mm. H_2O	21	31	34	43	47	62	83	95	103	108
pH inside	4.65	4.52	4.40	4.23	4.14	3.99	3.76	3.61	3.49	3.34
pH outside	4.65	4.50	4.37	4.19	4 09	3.92	3.69	3.53	3.39	3.23
pH inside minus pH outside	0	0.02	0.03	0.04	0.05	0.07	0.07	0.08	0.10	0.11
P.D. calculated, *millivolts*	0	1.0	2.0	2.5	3.0	4.0	4.0	5.0	5.5	7.0
P.D. observed, *millivolts*	0.5	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.5	6.0

Similar results were obtained, in the case of solutions of edestin, by Dr. Hitchcock, whose paper appears simultaneously with this note.[3]

SUMMARY AND CONCLUSIONS.

1. It had been noticed in the previous experiments on the influence of the hydrogen ion concentration on the P.D. between protein solutions inside a collodion bag and aqueous solutions free from protein that the agreement between the observed values and the values calculated on the basis of Donnan's theory was not satisfactory near the

[3] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

isoelectric point of the protein solution. It was suspected that this was due to the uncertainty in the measurements of the pH of the outside aqueous solution near the isoelectric point. This turned out to be correct, since it is shown in this paper that the discrepancy disappears when both the inside and outside solutions contain a buffer salt.

2. This removes the last discrepancy between the observed P.D. and the P. D. calculated on the basis of Donnan's theory of P.D. between membrane equilibria, so that we can state that the P.D. between protein solutions inside collodion bags and outside aqueous solutions free from protein can be calculated from differences in the hydrogen ion concentration on the opposite sides of the membrane, in agreement with Donnan's formula

A CUTANEOUS NEMATODE INFECTION IN MONKEYS.*

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PLATES 39 TO 52.

(Received for publication, December 21, 1921.)

INTRODUCTION.

While attempting to reproduce acute rheumatic fever in monkeys (*Macacus rhesus*), there were observed fever, subcutaneous nodules, and swellings about joints, which closely resembled some of the features of the disease in man. These features were first observed in monkeys inoculated with material from patients with acute rheumatism, but upon closer examination of the entire stock of animals, similar manifestations were occasionally encountered in normal animals and in some that had recovered from measles. In addition to subcutaneous nodules, the most striking lesion, upon casual observation, was a blister on the palms and soles, thought at first to be due to irritation from the disinfectant used in cleaning the cages. The keeper of the animal house stated that he had occasionally noted similar bullæ in monkeys imported in previous years. At first, no connection between these blisters and the subcutaneous nodules and periarticular edema was suspected, but later it seemed probable that all were dependent upon a single etiologic factor, a nematode. Search of the literature has failed to disclose a description of this disease in monkeys. As these animals are commonly used in laboratory experiments, it is thought advisable to make a note of the findings in order that other observers may be saved confusion.

Clinical Description.

The subcutaneous nodules most closely resembling similar nodules in children or adolescents with acute rheumatic fever were found over

* Read before the Society for Experimental Pathology, New Haven, December 28, 1921.

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the dorsal aspect of the hands and feet; they were easily missed unless the overlying hair was kept short by frequent shaving. The evolution of a single nodule was as follows: A circular area of subcutaneous edema from 4 to 10 mm. in diameter would appear. Careful palpation at times disclosed a small indistinct nodular thickening in the middle of the edematous area. After 2 or 3 days the edema would disappear and the nodule become more distinct. The smaller nodules, 1 or 2 mm. in diameter, were hard, globular, and definitely subcutaneous, so that the overlying skin could be easily moved over them; often they could be more readily detected by placing the hand or foot of the monkey between a light and the observer, when the overlying skin had a white or gray shining, translucent appearance. Larger nodules were not infrequently ovoid in outline and obviously more easily detected than the smaller ones (Fig. 1). Aside from edema, no objective signs of acute inflammation were grossly manifested in the skin or subcutaneous tissue; sometimes the animals gave evidence of moderate tenderness of the diseased area, at other times they seemed oblivious of any hurt. Following the initial appearance of the nodules they diminished in size so gradually that very little if any difference could be detected from day to day, but measurement every 3 or 4 days demonstrated a definite decrease. The smaller nodules persisted from 1 to 4 weeks; it was often difficult to determine definitely the exact day when they could no longer be detected. Small nodules of this type were seen most commonly over the dorsal aspects of the metacarpal or metatarsal bones, as if closely associated with the tendon sheaths in these regions; several were also seen over the metacarpophalangeal joints; and a few over the proximal phalanges. A few were found over the ulna just below the tip of the olecranon, and over the tendons in the lower third of the leg. Larger nodules, from 3 to 5 mm. in diameter, were occasionally found over the wrist or posterior aspect of the elbow joint. These larger nodules disappeared more slowly than the smaller ones; they never showed evidence of softening in the center.

A second type of subcutaneous lesion was found more frequently over the muscles of the arm (Figs. 2 and 3), forearm, and thigh, less frequently over the leg. It consisted of swellings of irregular size and extent. in which the process seemed to involve both the skin and sub-

cutaneous tissue.   At times these papules were flat, from 5 to 10 mm. broad and from 8 to 20 mm. long, and seemed to be from 2 to 3 mm. in depth; at other times they were narrow ridge-like thickenings, 2 to 3 mm. broad and 5 to 30 mm. long, sometimes straight, but more often comma-, L-, or horseshoe-shaped.   In some of these lesions indefinite nodules could be detected; in others the swelling assumed a diffuse serpiginous appearance.   The nodules, if present, often did not have the definite discrete feeling on palpation that was so characteristic of the lesions over the tendons of the hands and feet.   Occasionally, when the lesion was close to a superficially lying bony surface, discrete nodules were very distinct.   The more diffuse appearance of many of the papular lesions over muscular areas seemed to be due to a more intimate association between the nodule and overlying skin, and possibly more persistent edema than was present in the lesions over tendon sheaths and joints.   Occasionally larger single nodules similar to those about the joints were found over large muscle groups. Rarely, localized areas of firm cutaneous edema from 5 to 10 mm. in diameter were noted; they would persist from 2 to 5 days and quickly disappear without leaving any gross evidence of cellular infiltration. They resembled urticarial wheals more than subcutaneous rheumatic nodules.

A more extensive form of subcutaneous swelling was found about the ankles and wrists of some of the monkeys.   It was usually seen in only one extremity at a time, although in one instance both feet were involved simultaneously (Figs. 4 to 7).   It consisted of a diffuse thick, soft, edematous area, not sharply outlined; the overlying skin at times was glistening, due to the tension; but there was no other local sign of inflammation.   Occasionally the monkey favored the affected limb.   At times the edema was confined to the dorsum of the hand or foot (Fig. 8); again it was seen only about the internal or external aspect of the wrist or ankle joint.   It lasted from 2 to 10 days.   Once or twice the swelling disappeared after 2 or 3 days but reappeared after an interval of 3 to 5 days.

Attempts to obtain fluid by puncture of the swollen area yielded only a small amount of blood-tinged serum.   Puncture of the underlying ankle joint in two instances yielded only 1 or 2 drops of normal joint fluid that gave no bacterial growth on ordinary aerobic cultures.

No nodules were found in the subcutaneous tissues following the subsidence of this massive edema; but it was soon found that this type of swelling was always associated with a peculiar blister of the palms or soles in the immediate neighborhood of the swelling. The nature of these blisters gave the clue to the causation of all of the lesions described.

The most obvious pathologic alteration of the palms or soles was an elongated blister 3 to 5 mm. broad, and 2 to 10 cm. long, serpiginous in form as though something had burrowed between the horny epidermis and corium and deposited an irritating substance in its tract. Closer observation of the evolution of the bulla revealed at first a small oval blister about 3 by 5 mm. in size, filled with a blood-tinged serous fluid (Fig. 9); a day or two later it increased to an elongated serpiginous blister filled with similar material (Fig. 10); after another day the fluid became purulent, and the lesions had a yellow color. At this time a portion of the epidermis would be knocked off and the contents of the blister discharged; at times the bulla burst before the fluid had become purulent. In either event, the epidermis over the blister usually desquamated, leaving a smooth elongated serpiginous area with borders composed of ragged skin (Fig. 11). Gradually the denuded areas were covered with normal skin; and after 2 or 3 months the hand or foot showed no evidence of having been diseased. With the exception of the integument at the base and side of the nails, the only cutaneous area that has shown the bullous type of reaction has been the horny skin over the palms and soles, and between the fingers and toes; occasionally a blister was seen at the border of the palms extending 1 or 2 mm. into the neighboring hairy skin.

Occasionally by examination of the palms or soles with a hand lens a very fine serpiginous burrow was seen under the epidermis; it was from 0.3 to 0.5 mm. wide and several centimeters long, gray to brown in color, with no gross evidence of inflammation of the tissue at its side. At times the end of the burrow seemed to communicate with a collapsed blister. It is possible that this fine lesion, appearing as though a thread had been drawn in an irregular direction under the epithelium, is the earliest manifestation of the disease in the palms and soles—a stage before any tissue reaction has occurred.

Examination of the fluid obtained from the blisters revealed a lemon-shaped egg, characteristic of certain nematodes. Later adult worms, the diameter of a hair, about 2 cm. long, were obtained from several lesions.

Examination of the stools failed to reveal either the worm or eggs of this type. Microscopic examination of the tissue removed during life indicated that all of the lesions were due to the presence of a worm.

Temperature.

The temperature of the monkeys varied from 101.5–103.5°F., with an average afternoon temperature of about 1°F. higher than that of the morning. The temperature of the room exerted a certain influence, the temperatures of the monkeys being relatively low when the animal house was cold and high when it was warm.

In general, the presence of blisters and nodules did not influence the temperature of the monkeys. One animal having nodules over long periods, and also inoculated with human joint fluid on two occasions, showed only an occasional rise in temperature above 103°F. In a few instances, when infection of the blisters had occurred, accompanied by swelling and redness of the ankle or wrist, there was fever of 105°F.

Certain of the monkeys, both control and inoculated, at various times had unexplained short febrile bouts.

Blood Picture.

The blood picture resulting from this nematode infection was difficult to determine, as most of the monkeys at this time harbored another nematode of the genus *Œsophagostomum*, this latter being found encysted in the wall of the intestine, mesentery, and omentum. In the monkeys examined, which showed blisters and nodules, the leucocytes varied from 13,000 to 30,000 per c.mm. Leucocyte counts of monkeys not showing such lesions varied from 14,000 to 20,000 per c.mm. Eosinophilia, 7 to 30 per cent, was present in all monkeys examined.

Histopathology of the Subcutaneous Nodules.

Though the various clinical pictures are better understood after a study of the histopathology of the nodules of different ages, it is preferable to give a composite description of the lesions of various ages rather than to describe single nodules. The worm will be described in detail later. In all of the subcutaneous lesions in which it was found, it was from 35 to 55 microns in diameter and delimited by a hyaline cuticle staining pink with eosin-methylene blue, red with Van Gieson, and pale red to grayish red with methyl green-pyronine. This cuticle was at times the only worm structure that could be detected in older nodules; it seemed to be the portion of the parasite most resistant to the defensive agencies of the host.

In the youngest lesion, in which very little except edema of the skin and underlying tissues was clinically demonstrable, the area upon removal had a diffuse, gray, moist appearance, and no definite nodule could be detected. Microscopic examination of such an area usually revealed a single cross-section of the worm in each section; the parasite was surrounded by a collar of epithelioid cells and polymorphonuclear eosinophils; the total thickness of the collar was from two to five cells (Figs. 12 and 13). Small blood vessels nearby showed a marked perivascular reaction made up of actively dividing epithelioid cells (endothelial leucocytes) and polymorphonuclear eosinophils; vessels situated at least 10 mm. from the worm often showed this perivascular reaction (Fig. 14). The surrounding connective tissue showed distinct edema with scattered eosinophils (Figs. 12 and 13). In some slightly older lesions the worm and collar of cells were surrounded by a narrow circular area filled with fibrin; this in turn was encompassed by an area made up of a loose network of fibrin and cells with branching cell bodies and pale vesicular nuclei (Fig. 14). Edema, perivascular reaction, and diffuse infiltration with eosinophils were present in the surrounding tissue of all of the early lesions. In an area slightly older than the one just described many newly formed thin walled blood vessels were seen; also a few polynuclear cells with two, four, eight, or more closely packed, pale vesicular nuclei and very little cell body; they were probably young giant cells.

In those lesions, however, in which a distinct nodule from 2 to 4 mm. in diameter was evident at the time of earliest observation, microscopic examination revealed several coils and cross-sections of the parasite in each section; it seemed as though the worm were confined to a smaller area with consequently a more intense local reaction (Figs. 15 and 16).   Some coils of the worm were surrounded simply by coagulated blood, others by a collar of cells similar to that previously described; often many of these cells had deep staining pycnotic nuclei as if they had been injured by some toxic agent; still other sections of the parasite were surrounded by epithelioid cells, lymphocytes, eosinophils, and giant cells.   In two nodules this area of worm, hemorrhage, and cell reaction was surrounded on all except one side, or peduncle, by a zone of fibrillar and granular material into which were growing many young actively dividing cells with pale, vesicular nuclei and cell bodies with long branching processes; these young cells could be seen arising from the connective tissue surrounding the nodule.   This area of actively growing cells closely resembled the edge of a tissue culture (Fig. 16).   The portion of the lesion called the peduncle was made up of many newly formed capillaries and cells with large, pale, vesicular nuclei.   As one passed towards the normal connective tissue these cells approximated more and more the appearance of the fixed connective tissue elements. In none of the lesions of this age was there much capsule formation, although in some areas a mosaic of spindle cells with large pale nuclei could be detected; methyl green-pyronine stained sections showed plasma cells most numerous in this zone.   Practically all of the small blood vessels within a radius of 3 to 5 mm. showed the marked perivascular reaction previously mentioned; about some of them many closely packed plasma cells were visible, and about others were numerous eosinophils.   In fact, eosinophils were present throughout all portions of the sections, both in the zone of active cell proliferation and in the surrounding edematous tissue.

In a slightly older lesion the coils of worm were immediately surrounded by mononuclear cells in many of which the nuclei were deep staining and pycnotic; next to this zone there were a number of large giant cells mixed with many eosinophils and a moderate number of epithelioid cells (Figs. 17 and 18).   Scattered throughout the nodule

were several areas made up largely of newly formed capillaries distended with blood. At the periphery of the nodule definite early capsule formation was present; outside of this the blood vessels still showed marked perivascular reaction, and small foci of edema were seen in the connective tissue in contradistinction to the diffuse edema seen in the earlier lesions. A nodule having approximately the same general structure showed in only eleven of the sections easily distinguishable cross-sections of the parasite; nearby were annular hyaline structures of the same thickness, diameter, and staining reaction as the cuticle of the worm; occasionally these rings were filled with mononuclear wandering cells. No large giant cells were seen in this lesion, but there were several smaller cells with eight to fifteen closely packed oval or round vesicular nuclei and a distinct cell body of a red, finely granular appearance in methyl green-pyronine stained sections. These cells closely resembled the large cells seen in the Aschoff bodies in the hearts of patients dying in the acute stages of rheumatic fever. In fact, in this nodule it would have been difficult to differentiate the histological structure from that of early subcutaneous nodules in children with rheumatic fever, had no sections of the worm been found.

In nodules 2 weeks or more old capsule formation was more marked, many plasma cells were found immediately under the capsule, and young actively dividing cells were comparatively less numerous in the interior of the lesion. Distinct cross-sections of worms were found in only three out of seven old nodules examined; and in these only a few of the sections contained a single cross-section of worm. In one other lesion hyaline ring-like structures resembling the cuticular membrane were seen. In all of the older lesions containing a portion of the worm there were many large giant cells often surrounding the parasite like a ring; in nodules containing no worm they were less numerous and were absent in two nodules with very thick capsules (Fig. 19). It seems probable that these giant cells function to remove the parasite and disappear as soon as this task is completed; this view is substantiated by their being most numerous in the nodules in which the worm is undergoing disintegration and by their gradual disappearance in lesions in which no remains of the parasite can be found. The presence in the younger lesion of polynuclear cells with two, four,

eight, or more nuclei and the fact that these cells are not far from the worm suggest that the giant cells are formed from the young actively growing cells that have invaded the lesion. A study of many lesions of various ages revealed this giant cell development from cells containing two nuclei to old cells with many nuclei peripherally situated.

Nodules a month or more old consisted chiefly of fibrous tissue in which the cells were more densely arranged at the periphery (Fig. 19). Eventually these nodules completely disappear, for none could be found post mortem in several monkeys killed 3 or 4 months after the clinical disappearance of multiple subcutaneous lesions.[1]

Histopathology of the Palmar and Plantar Lesions.

The chief site of activity of the nematode in the palms and soles was in the epidermis, with only slight accompanying reaction in the superficial portion of the corium. The principal portion of the epidermis involved was the stratum mucosum below the stratum lucidum and above the stratum germinativum. An early lesion taken from a place where the worm was grossly detected, and cut so that several cross-sections of the parasite were present in a single section, showed different degrees of reaction. Occasionally a single cross-section of the worm was seen in the stratum mucosum of the epidermis with the neighboring epithelial cells appearing almost normal. In other places, close by, small areas slightly larger than the diameter of the parasite were seen filled with granular material, red blood cells, and degenerated epithelial cells (Fig. 20). Generally the area of reaction about the parasite was much larger and consisted

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[1] Incidentally another type of subcutaneous nodule was found in several monkeys, most often about the extensor aspect of the elbows. Grossly they were 1 or 2 mm. in diameter and consisted of a small brown central portion surrounded by a tough white membrane. Microscopically the central portion was made up of six-sided cells with a thick membrane containing a more or less homogeneous material; the entire cell stained deep blue with methylene blue-eosin. They were thought to be vegetable cells making up possibly the points of thorns or splinters of wood. Surrounding the mass of vegetable cells were a few foreign body giant cells and a dense fibrous tissue capsule. Nodules of this type remained constant in size and never showed the perinodular and subcutaneous edema noted about the lesions containing a worm.

in cross-section of a vesicular area 1 mm. thick and 2 to 4 mm. in breadth.   In early lesions before rupture of the blister, the worm was usually found close to the stratum germinativum, which in most places seemed to be intact, but occasionally small areas of this stratum were missing and the blister was in direct contact with the superficial layers of the corium.   In these places it was not unusual to see capillaries coursing upward through the corium and emptying into the blister.   In the superficial layers of the corium immediately under the blisters there was a definite increase of cells, usually mononuclear in type, but in places small groups of polymorphonuclear leucocytes were seen.   Rarely did the small blood vessels of the corium show the perivascular reaction which was so prominent a feature in the subcutaneous nodules.   Occasionally there was definite edema of the subcutaneous tissue surrounding the blister, with a moderate number of polymorphonuclear eosinophils scattered through the edematous area.

The contents of the early unbroken blister consisted of amorphous granular material, many red blood cells, and a few leucocytes, both mononuclears and polymorphonuclears; epithelial cells in various stages of degeneration were also present.   In older blisters there were many polymorphonuclear leucocytes but fewer red blood cells and epithelial cells.   In most of the unbroken blisters it was possible to find the characteristic eggs of the nematode, which in all instances contained fully developed embryos.   At times, surrounding the eggs there was a thin, pink-staining, hyaline, annular structure, irregular in outline and two or three times the diameter of the egg.   Some sections of the blisters contained the adult parasite, others did not; if present the number of cross-sections of the worm in a single section varied from one to six or eight.   In some lesions excised following an unsuccessful attempt to remove the worm intact, small portions of the parasite were found winding through the epidermis.   This peculiar winding made it difficult to obtain many complete uninjured specimens of the nematode from the blisters.   Sections of skin removed after the blister had ruptured showed the stratum corneum and stratum lucidum separated from the stratum mucosum by an open space; usually the stratum germinativum was intact but occasionally it was missing for a short distance.   The fact that the deepest layer of

epithelium was rarely completely destroyed and that there was little destruction of the superficial layers of the corium explains the usual absence of deep ulceration after the desquamation of the top of the blisters; the germinal layer of the epithelium was in condition quickly to repair the damage.

It is evident that the two types of lesions differ in more than one respect. In the subcutaneous nodule the nematode is smaller in diameter, and probably in length, although this point cannot be definitely determined because of the impossibility of obtaining specimens of the parasite except in sections from the subcutaneous tissue. The anatomy of the worm found in the nodules was much simpler than that of the female nematode found in the cutaneous blisters (Figs. 21 to 23). No bodies resembling eggs were ever seen in the worms in the subcutaneous lesions or in the tissue immediately surrounding them; on the other hand, eggs in various stages of development were found in all of the parasites in the epidermal blister, and eggs containing embryos were constantly present in the blisters. In both the subcutaneous lesion and the epidermis the first effect of the parasite seemed to be necrosis of the cells immediately surrounding it, followed by vesicle formation with a rapid filling of the vesicle by red blood cells.

The hemorrhage surrounding the female worm in the epidermal burrows and many of the worms in the subcutaneous tissue is probably due to some toxic substance secreted by this parasite. Schwartz (1) lately showed that certain nematodes contain substances that inhibit the coagulation of blood to a marked degree and appear to be similar to leech extract. This toxic substance is evidently of importance in insuring the worm a proper supply of food, for the adult female lives literally in a pool of blood. The finding of hemorrhage about many of the nematodes in the subcutaneous nodules is a point in favor of the theory that the worms in both the epidermis and subcutaneous nodules belong to the same species.

In the subcutaneous tissue the intensity of the local response was so great that the parasite was prevented from wandering farther; on the contrary it seems to have been quickly killed, firmly encapsulated, and subsequently removed like any other easily phagocytable foreign body. In other words, the response of the tissues of the host in this re-

gion was sufficient to prevent the parasite from continuing its exist-
ence. The conditions in the epidermal lesion, on the other hand, were
more favorable for the parasite. By continuing its existence in the epi-
dermis during the egg-laying stage and constantly invading new skin
areas, the female worm found favorable conditions for the deposition
of its eggs and their extrusion into the external world where they
might infect new hosts. The reaction in the epidermis never seemed
to be sufficiently intense to kill the worm. On the contrary, the ease
with which the blister burst and scattered its contents rendered espe-
cially easy the dissemination of the eggs. There is here a condition of
almost perfect parasitism in which the injury inflicted by the nematode
on the host is not sufficient to impair seriously the health of the host,
but in which the local injury in the palms and soles is of such a nature
as to favor the continuation of the existence of this peculiar nematode.

It has been impossible for us to determine the life history of this
nematode. It is possible that some of the worms found in the
subcutaneous lesions were males; but if they were males, one cannot
state whether they migrated to the subcutaneous tissue after fertiliz-
ing the female or whether they reached this tissue before having
completed their sexual function. We have examined post mortem
several monkeys with active skin lesions and, with the exception of
one lymph node containing a larval form and tubercle-like nodule,
have found no lesions resembling the skin blisters or subcutaneous
nodules in any of the viscera or muscles. In so far as our observa-
tions go they indicate that practically the entire lesion producing
activity of this nematode is in the epidermis and subcutaneous tissue.

Attempts to hatch the eggs in moistened filter paper kept at body
temperature for 2 months have failed to induce the embryos to mi-
grate from the shells. The injection of the eggs into the skin of
the palms and soles of monkeys and into the subcutaneous tissue
of the extremities has not been followed by lesions like those ob-
served in naturally infected animals; there was slight local reaction
lasting 3 or 4 days, following which there was practically no gross
evidence of the introduction of any irritating substance. The fact
that fully developed embryos can be caused to emerge from the eggs
obtained from skin blisters by simple mechanical pressure shows
that they are probably in condition to continue their life's activity

immediately when brought under favorable conditions. It seems most probable that the digestive juices of the stomach or intestine act upon the opercular plug and allow the embryo to escape; it is well known that eggs of this character are usually hatched in the stomach or intestine, after which the embryos continue their activity by burrowing through the wall of the gastrointestinal tract and migrating to various parts of the body. An intermediary host is therefore not necessary for the continuation of this type of parasite.

We have searched in vain for the characteristic eggs in the feces of twenty monkeys that had shown skin lesions and in the entire intestinal contents removed post mortem from several other monkeys; Kofoid's (2) brine concentration method was employed to obtain the material for examination. The eggs of another common intestinal parasite, an *Œsophagostomum*, were found in the feces of most of the monkeys examined. It seems probable, therefore, that the only manner in which the *Trichosoma* eggs are extruded from the body of the monkeys is from the skin lesions. In so far as we are able to determine, this method of dissemination of eggs has not been previously described for nematodes.

It is not unusual to find lesions of the skin and subcutaneous tissue caused by animal parasites. Ground-itch is well known to be due to invasion of the skin by the larvæ of *Ancylostoma duodenale*. Looss (3) states that a similar condition may result from infection with larvæ of *Strongyloides stercoralis*. Subcutaneous tumors in man may be caused by *Filaria bancrofti*, *Loa loa*, *Agamofilaria georgiana*, and *Rhabditis niellyi*. The female guinea-worm (*Dracunculus medinensis*) burrows in the subcutaneous tissues and discharges young larvæ from her body, through an ulcer in the skin to the outer world. This is the closest parallel we can find in the literature to the action of our *Trichosoma* in which eggs are deposited in an epidermal blister before being discharged from the body of the host.

A somewhat similar microscopic lesion of the epidermis is seen in larva migrans, or creeping eruption, which is due to burrowing in the epidermis by the larvæ of a *Gastrophilus* (bot-fly). The gross picture, however, is different; it consists of a migratory red line in the skin from 1 to 9 mm. broad, in the center of which is a fine line due to the burrow. Minute vesicles are occasionally seen along the course of

the red line, but the entire lesion does not become bullous or purulent (4).

Castellani and Chalmers (5) report a rare skin disease of unknown etiology found in natives of Ceylon and South India; it is called dermatitis macrogyrata, and is characterized by the presence on the palms of the hands of one or two very large gyrations formed by scaling and crusty lesions. On removing the crusts and scales a broad shallow furrow can be seen situated in the epidermis. Neither fly larvæ nor fungi are found in the lesions. The photograph of the condition in Castellani and Chalmers' book resembles very closely the lesions seen in our monkeys after the removal of the top of the blister. It is interesting to conjecture upon the possibility of the two conditions being due to a common cause, inasmuch as *Macacus rhesus* comes from the same part of the world as the patients in whom dermatitis macrogyrata is found.

Description of the Nematode.

Female.—Only a few intact specimens of the female worm were obtained from the blisters on the hands or feet. Many broken specimens were secured, all of which contained eggs. The worms were all of practically the same size; the head and esophagus were of about one-half the diameter of the portion of the body containing eggs; the body gradually increased in diameter from immediately behind the head to the tail. Measurements were as follows:

Head: Length 42 microns; width 52 microns.
Diameter of worm at posterior end of esophagus: 100 to 110 microns.
Diameter of posterior portion of body: 200 microns.
Length of body: 22 to 24 mm.
Esophageal portion of body: One-sixth of the entire length of the worm.

In unstained specimens fixed in alcohol and mounted in glycerol, the nematode showed a hyaline cuticle with slight annular striation. Beginning about the middle of the body were seen numerous highly refractile fine papillæ, arranged in two broad bands towards the posterior portion of the body. The cuticle about the head was about twice the thickness of that elsewhere; the head was thus slightly ovoid in shape. No teeth could be detected. The esophagus con-

sisted of a single chain of from 85 to 95 cells with the lumen in the center. The digestive tube then showed a slight sacular dilatation, followed by a long intestine which was arranged in loose coils in the posterior half of the body. The anus was terminal. The posterior end of the body was bluntly conical.

The vulva was situated just posterior to the termination of the cuboidal esophageal cells. There was a single ovary at the posterior end of the body. Between the ovary and the vulva the eggs could be seen in the uterus gradually developing into definite embryos and taking on the characteristic brown, lemon-shaped shell with round canal at each end covered by an opercular plug.

The eggs measured from 40 to 42 microns in breadth and 67 to 70 microns in length, had a thick highly refractile hyaline shell, usually brown in color, and contained a coiled embryo, which upon pressure could be caused to emerge through the opening at one end (Fig. 24).

The embryo had bluntly rounded ends and was filled with a uniform colorless material containing a group of highly refractile droplets toward the posterior end.

In sections of the epidermis stained with eosin-methylene blue, the female parasite was surrounded by a pink hyaline cuticle. Inside of this were two single rows of blue-staining cuboidal muscle cells; each row occupied about one-third the circumference of the worm (Figs. 25 and 26). Between these rows of cells and slightly overlapping the ends were more loosely arranged pink-staining cells with long communicating processes, considered as nerve cells. In all cross-sections a flattened annular structure, evidently the intestine, was seen lined with a single layer of cuboidal cells. In a number of instances the lumen of the intestine contained a few erythrocytes.

In some sections the remainder of the body was filled with a single cross-section of the uterus with maturing eggs (Fig. 26). Elsewhere, there were two or three cross-sections of the generative organs. Some contained young eggs, others consisted of a thin hyaline ring enclosing numerous long flat deep blue-staining bodies radially arranged and usually pointing towards the center. Mesial to these, there was a loose fibrillar meshwork with a few similar elongated bodies. In a few sections large cells resembling young eggs were seen in the

center of this organ, and in several longitudinal sections this organ (supposed to be the receptaculum seminis) was seen to communicate directly with the egg-containing structure (Figs. 25 and 27).

No sections were obtained through the esophagus of the female worm. In all of the sections above described the diameter of the worm was from 140 to 160 microns.

Worm in Subcutaneous Nodules.—In one series of sections the entire worm was included (Fig. 28). It was fairly uniform in size, and measured from 35 to 45 microns in diameter. It was surrounded by a thin hyaline pink-staining cuticle, inside of which was a single ring of cuboidal cells. In some places this ring of cells was almost continuous; elsewhere, it was broken into two segments each occupying about one-third the circumference of the worm, with one or two larger cells between the ends of the segments. In many cross-sections the remainder of the worm was filled with granular amorphous pink-staining material. On longitudinal section, one end of the worm was made up of cuboidal cells filling the entire parasite. These resembled the esophageal cells seen in the female, and evidently corresponded to the same pink-staining areas described in cross-sections. In other sections, most often towards the posterior end of the worm, were seen two circular structures made up of elongated flat deep blue-staining bodies similar in shape and size to those seen in the receptaculum seminis of the female worm. In longitudinal sections these flattened bodies were pointed antero-posteriorly. No lumen or structure resembling an intestine was seen in this specimen.

A fresh serpiginous lesion after excision showed on gross examination a small waxy line. In these sections many cross-sections of a worm were seen; they were larger and the worm was evidently more mature than the one described above; measurements of several cross-sections showed a diameter of about 55 microns (Figs. 29 to 31). There was a pink hyaline cuticle inclosing a single ring of cells. This ring was divided into four segments, two of which contained cells with rounded deep blue-staining nuclei, each segment occupying about one-third the circumference of the worm; the other two were made up of pink-staining cells resembling the nerve cells of the female worm. Back of the esophagus were two distinct structures in the body of the

worm. One was an elliptical ring lined with a single layer of flat cells, evidently the intestine. The other was a round body occupying about one-half the diameter of the worm and in places filled with small cells with solid deep blue nuclei; in other places the nuclei were arranged concentrically about a small lumen; in still other sections the lumen was larger and surrounded by a single layer of cuboidal cells. At the head end of the worm longitudinal sections showed large cuboidal esophageal cells, and cross-sections showed these cells occupying the entire diameter of the parasite inside of the muscle and nerve cells.

It was thought probable that this was a male worm. The muscle and nerve cells, esophageal cells, and intestine resembled similar structures of gravid female worms in the epidermis. The genital tract, however, was different.

The first of the worms described in the subcutaneous tissue was evidently a larval form, as neither the intestinal nor genital tract was well differentiated. The second was more mature, with a genital tract distinctly different from that of the adult female. Both forms were seen in other sections. It seems plausible, therefore, to conclude that the subcutaneous nodules contained both immature larvæ and fairly well developed male worms.

Classification of the Nematode.

The parasite is clearly a member of the nematode superfamily Trichinelloidea Hall, 1896a, family Trichinellidæ Stiles and Crane, 1910. Hall (6) divides this family as follows:

"1. Male with 1 spicule or at least with a copulatory sheath ..... Trichurinæ.
  "Male without spicule or copulatory sheath........................2.
"2. Eggs spherical without true egg shell; ovoviviparous; males not parasitic in females; adult worms in digestive tract.................Trichinellinæ.
  "Eggs variable in shape and with true, thick shell and opercular plugs; oviparous; males parasitic in the uterus of females; worms in kidney pelvis ureters or in the urinary bladder...............Trichosomoidinæ."

On characters of the female, namely size and shape of body and location in host, our worm can be safely eliminated from the sub-family Trichinellinæ. As an examination of ten gravid females failed

to reveal the presence of males in the uterus, this worm is apparently to be eliminated from the subfamily Trichosomoidinæ. Hall (1916a, p. 19) divides the remaining subfamily, Trichurinæ, as follows:

"1.   Anterior esophageal region of body very slender and longer than the posterior portion, which is much thicker and contains the reproductive organs..*Trichuris.*
"Anterior esophageal portion of body shorter than, rarely equal to, the posterior portion, which is only slightly thicker...................2.
"2.   Worms with spicule; in digestive tract or urinary bladder, ducts, etc. ....*Capillaria.*
"Worms without spicule; in liver........................*Hepaticola.*"

This key clearly eliminates the genes *Trichuris* from consideration. There remain for consideration the following closely allied genera: *Capillaria* Zeder, 1800a, and *Hepaticola* Hall, 1916a. *Capillaria*, *s.l.* (synonym *Trichosoma*) is divided by various authors into *Capillaria*, *s. str.*, *Thominx* Dujardin, 1845a, and *Calodium* Dujardin, 1845a, which are variously recognized as genera or as subgenera.

It will be noticed that to classify a species of Trichurinæ in its proper genus and subgenus, it is absolutely essential to examine the male worm, and this sex we have thus far been unable to find. Any generic classification is, therefore, only provisional and will of necessity be subject to possible change as soon as the male is studied.

The old collective genus of this group is *Trichosoma* Rudolphi, 1819a, which is *Capillaria* Zeder, 1800a, renamed. If we classify the worm in *Capillaria*, *s. str.*, *Hepaticola*, *Thominx*, or *Calodium*, it is clear that we predicate certain anatomical characters upon the basis of which the modern classification is founded; accordingly, we introduce an element of confusion in anatomy. By placing the worm in the collective genus *Trichosoma* (which is *Capillaria sensu lato*) we avoid all complications as to detailed points in anatomy, but make it inevitable that later the worm will be transferred to one of the modern and more restricted generic groups. Both courses are open to criticism, but it seems that the latter course is the more conservative, since changes of names are more easily made than changes in erroneous conceptions as to anatomy; further, *Trichosoma* gives the approximate, though not the taxonomically exact, location of the worm. Accordingly, the parasite is provisionally referred to *Trichosoma* pending the finding of the male.

As it has been impossible to find any reference to a dermal tricho-some in monkeys, we assume that the species is new and we propose for it the name *Trichosoma cutaneum*, 1922.[2]

SUMMARY.

A number of monkeys (*Macacus rhesus*) were found to be infected with a nematode which gave rise to several types of skin lesions, subcutaneous nodules, edema about the joints, and elongated ser-piginous blisters of the palms and soles.

In the subcutaneous nodules were found larval forms of the nema-tode and possibly adult male forms.

The reaction about these worms consisted of proliferation of fixed cells, and invasion of eosinophils, with subsequent presence of giant cells, young blood vessels, and finally capsule formation; eventually the worms were killed, eliminated, and the nodule disappeared.

In the skin of the palms and soles the adult female worm burrowed in the epidermis, producing an elongated serpiginous blood blister that eventually became purulent. In this blister the worm laid her eggs; and by the bursting of the blister the eggs were discharged into the outer world and placed in a position to infect new hosts. The reaction in the epidermis was evidently not severe enough to interfere seriously with the health of the host or with the continuation of the egg-bearing period of the female parasite. This condition of almost perfect parasitism is an ideal one for the continuation of the life of this species of nematode. In so far as we are able to determine this is the first description of a nematode that lays its eggs in the epidermis.

The provisional name of the parasite is *Trichosoma cutaneum*, 1922.

[2] In seeking to identify this parasite in literature we have consulted the Stiles and Hassall nematode catalogue (Stiles, C. W., and Hassall, A., *Bull. Hyg. Lab*, *U.S.P.H.*, *No. 114, 1920*), Stossich (Stossich, M., *Boll. Soc. adriat. sc. nat. Trieste*, 1890, xii, 3), Travassos (Travassos, L., *Mem. Inst. Oswaldo Cruz*, 1915, vii, 146), and various other publications, but have found no record of cutaneous *Capillaria* in monkeys; neither has a record of this kind been found in the host catalogues of parasites in the Zoological Division of the United States Public Health Service and of the Bureau of Animal Industry.

For valuable assistance in this portion of the work and many helpful suggestions we are indebted to Professor Charles Wardell Stiles of the United States Public Health Service.

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2. Kofoid, C. A., *J. Am. Med. Assn.*, 1918, lxxi, 1557.
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4. Knowles, F. C., *J. Am. Med. Assn.*, 1916, lxvi, 172.
5. Castellani, A., and Chalmers, A. J., Manual of tropical medicine, London, 3rd edition, 1919, 2210.
6. Hall, M. C., Nematode parasites of mammals of the orders Rodentia, Lagomorpha, and Hyracoidea, Washington, 1916, No. 2131, from the *Proceedings of the United States National Museum*, 1916, 1, 1.

EXPLANATION OF PLATES.

PLATE 39.

FIG. 1. Showing large and small subcutaneous nodules in the dorsum of the hand.

FIG. 2. Showing an elongated papular area above the left elbow.

FIG. 3. Showing a broad papule over the tendon of the triceps.

PLATE 40.

FIGS. 4 and 5. Showing simultaneous swelling of the right ankle and left foot.

PLATE 41.

FIGS. 6 and 7. Lateral view of the ankle and foot shown in Figs. 4 and 5.

PLATE 42.

FIG. 8. Showing pitted edema of the dorsum of the left hand.

FIG. 9. Showing a very early small blister.

FIG. 10. Showing an elongated blister at the base of the great toe.

PLATE 43.

FIG. 11. Showing ragged edged desquamating areas over the plantar region.

PLATE 44.

FIG. 12. Early subcutaneous lesion showing a slight collar of cells about the worm and marked infiltration of eosinophils in the surrounding edematous tissue. × 230.

FIG. 13. Early subcutaneous lesion showing a slightly more marked collar of cells about the parasite and eosinophils in the neighboring edematous tissue. × 450.

PLATE 45.

FIG. 14. Early subcutaneous lesion showing the worm surrounded by fibrin and a few cells, in a tissue space, and perivascular reaction at some distance from the worm. $\times$ 55.

FIG. 15. Older subcutaneous nodule containing two cross-sections of a worm. *W*, worm (two cross-sections) surrounded by an intense cellular reaction; *PV*, perivascular reaction. $\times$ 85.

PLATE 46.

FIG. 16. Large globular subcutaneous nodule containing many sections of a worm, surrounded by a hemorrhagic zone. *HZ*, hemorrhagic zone; *CZ*, clear zone containing granular material and a few cells; *Y*, zone of young actively dividing cells; *P*, pedicle of the nodule containing many newly formed blood vessels; *PV*, perivascular reaction. $\times$ 85.

PLATE 47.

FIG. 17. Older subcutaneous nodule containing a single cross-section of a poorly staining worm. *W*, worm; *G*, giant cells surrounding worm; *C*, capsule; *PV*, perivascular reaction. $\times$ 85.

FIG. 18. Higher power of the portion of Fig. 17 showing the worm. *W*, worm; *G*, giant cells. $\times$ 450.

PLATE 48.

FIG. 19. Old subcutaneous nodule. $\times$ 85.

PLATE 49.

FIG. 20. Low power section of an early unbroken plantar blister containing a female nematode. *W*, worm; *WI*, cross-section of worm with very little necrosis of cells; *V*, microscopic vesicle containing only cell detritus. $\times$ 48.

PLATE 50.

FIG. 21. Unstained specimen of female *Trichosoma cutaneum* obtained from a palmar blister. *H*, head; *P*, posterior portion of body; *V*, vulva. $\times$ about 9 5.

FIG. 22. Head and esophagus. *H*, head; *E*, esophagus surrounded by cuboidal cells; *V*, vulva, from which eggs containing embryos are being expelled. $\times$ 37.

FIG. 23. Posterior end of the body containing the ovary and young ova. $\times$ 37.

PLATE 51.

FIG. 24. Eggs of *Trichosoma cutaneum*, each containing a well developed embryo. $\times$ 400.

FIGS. 25 and 26. Cross-section of a female *Trichosoma cutaneum* from a palmar blister. *M*, muscle cells; *N*, nerve cells; *I*, intestine; *O*, ovary; *U*, uterus containing partially matured eggs; *RS*, receptaculum seminis; *E*, empty egg-shell. × 410.

FIG. 27. Longitudinal section of a female parasite from a palmar blister. *RSO*, union of receptaculum seminis and ovary; *RS*, receptaculum seminis; O, ovary. × 400.

PLATE 52.

FIG. 28. Young worm in a subcutaneous nodule. *E*, esophageal cells, longitudinal section; *F*, cross-section of esophageal cells; *C*, cross-section of the posterior portion of the body. ×450.

FIGS. 29 to 31. Male (?) form of nematode in a subcutaneous nodule.

FIG. 29. Esophageal cells (*E*), longitudinal section. × 450.

FIG. 30. *E*, esophageal cell, cross-section; *I*, intestine; *G*, genital tract containing lumen. × 450.

FIG. 31. *M*, muscle cells; *N*, nerve cells; *I*, intestine; *G*, genital tract (testis?). × 450.
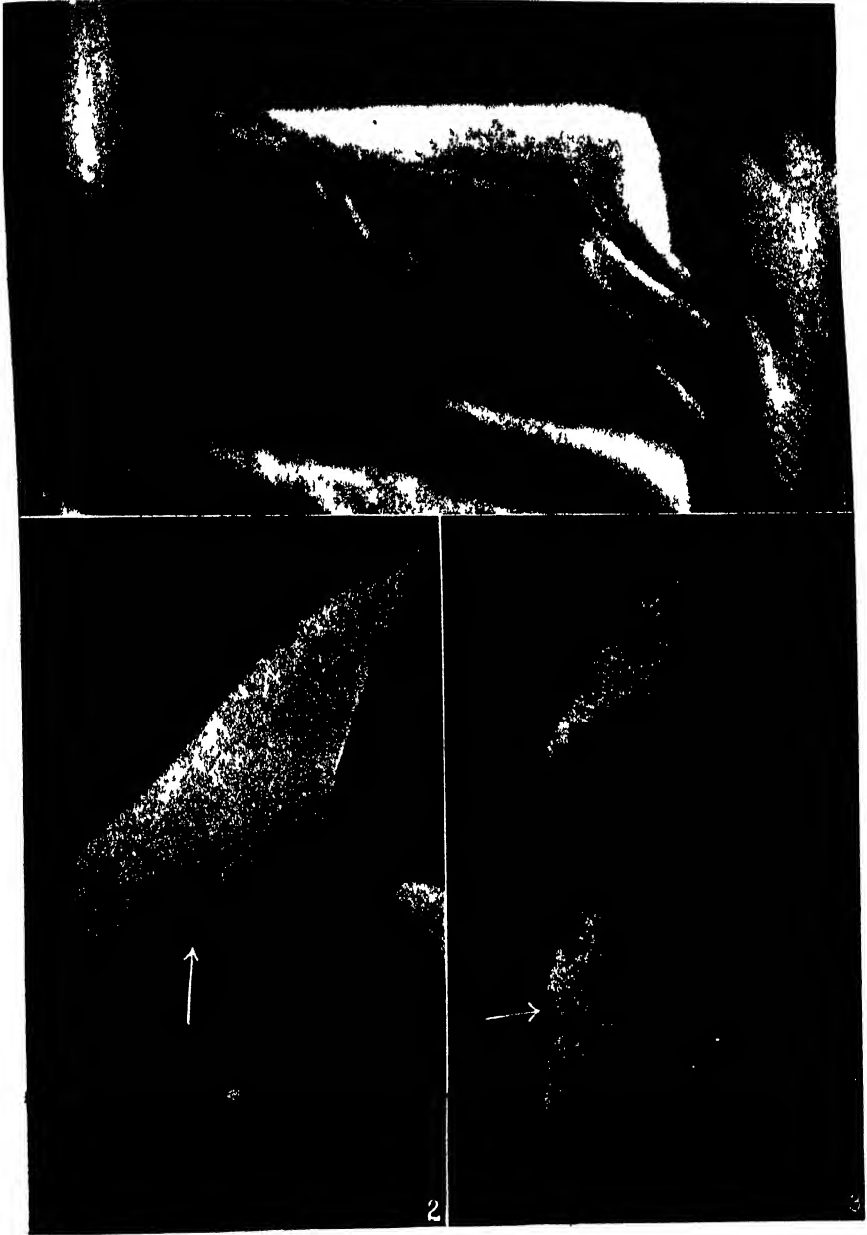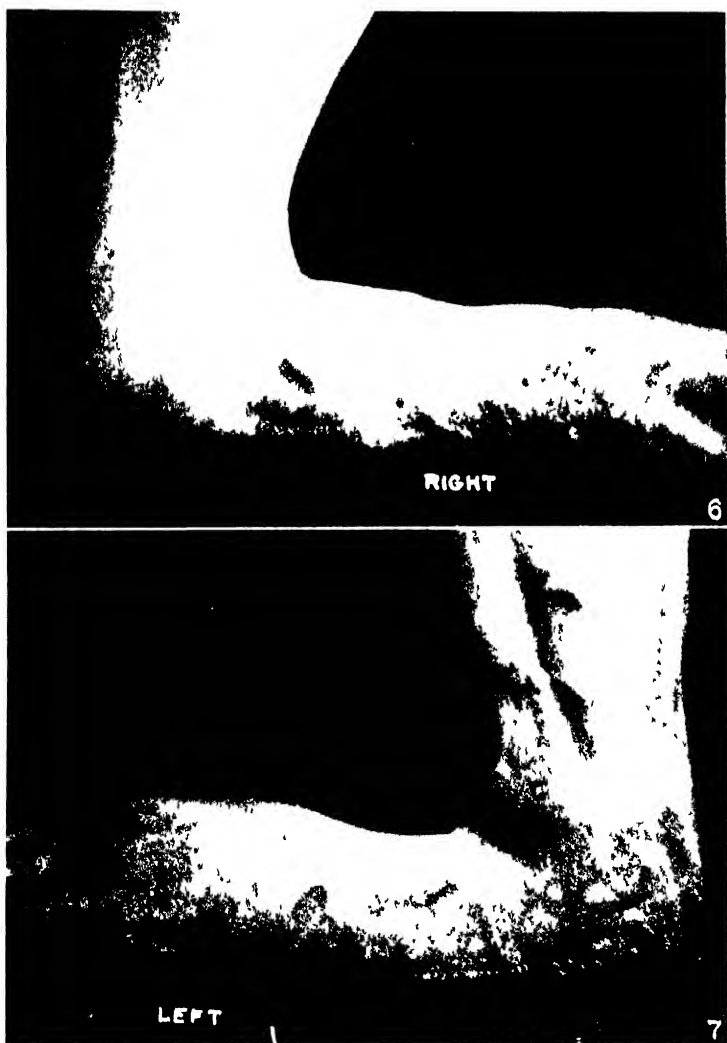
(Swift, Boots, and Miller   Cutaneous nematode infection in monkeys)

(Swift, Boot, and Miller: Cutaneous reaction of limbs of immune monkey.)

PLATE 46.

17

(Swift, Boots, and Miller: Cutaneous nematode reaction in monkeys.)

PLATE 48.

19
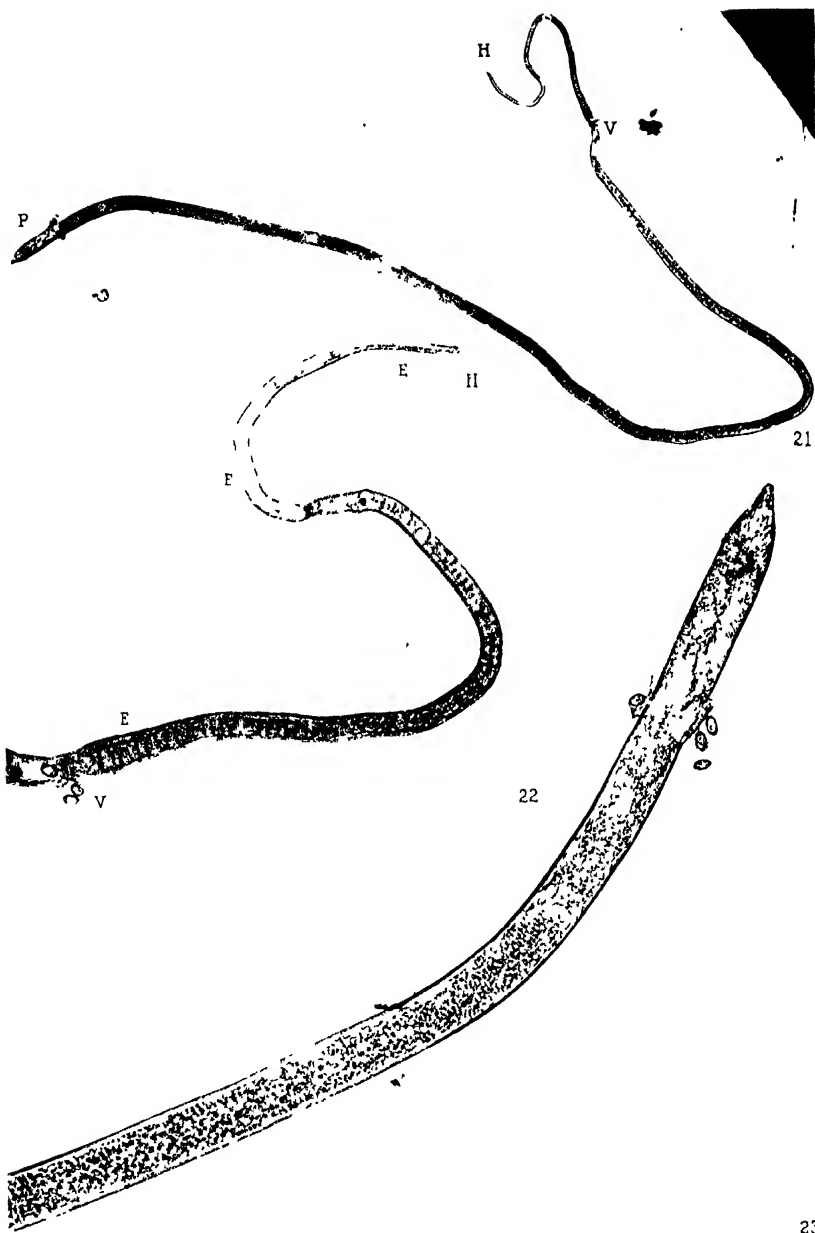
THE FOOD REQUIREMENTS OF CHILDREN.

III. FAT REQUIREMENT.

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The proper amount of fat which is required in the diet during growth has not been and cannot be established since the exact rôle of fat has not yet been determined. Until recently it has been generally supposed that fat did not play any specific part in nutrition, but like carbohydrate, served as a source of energy and as a sparer of protein. In fact, many observers have believed that fat and carbohydrate are practically interchangeable in the diet. Because of this belief little attention has been given to the amount of fat which is desirable in the diet, except in the case of infants. Recent investigations, however, have shown that fat is of greater importance than was formerly believed and that it has specific functions quite apart from those which it shares with the carbohydrates. It has been shown that especially during growth fat is essential. There are now admitted to be several reasons for regarding fat as an important constituent of the diet of children.

Functions of Fat.

Fat is the purveyor of the fat soluble vitamin which has been proven to be essential for health and normal growth. A fuller discussion of this particular vitamin will be taken up later with the general subject of food accessory substances. How much of this vitamin should be provided for the child is not yet known. No one of the vitamins has as yet been isolated, consequently the chemical nature and composition of them are unknown. It is, therefore, only possible to express the vitamin content of the different foodstuffs in a relative way, that is, by comparing the value of any one food with some other

used as a standard. For example, cod liver oil is used as a standard of comparison for foods containing the fat soluble vitamin A, while yeast serves as the standard for those containing the water soluble B and orange juice for those containing the antiscorbutic or vitamin C. The vitamin content of most articles of food is, however, not constant but is influenced by a number of conditions. For example, summer milk from pastured animals has been shown to have a much higher vitamin content than winter milk from stall fed animals which received no fresh fodder.

In view of these facts it is not possible as yet to state how much fat should be given to a child in order to supply an adequate amount of fat soluble vitamin. From the evidence which has been obtained it seems quite possible that the amount of fat needed for this purpose is comparatively small. However, in order to insure normal growth, it is safer to supply generous amounts of the fats known to contain this substance. The fat soluble vitamin is most abundant in cod liver oil. Among the usual foodstuffs the richest sources are milk fats, egg yolk and certain animal organs—liver, kidneys, etc. This vitamin is also found in the leafy vegetables, but, since a child can take only a comparatively small quantity of these in the diet, it is not wise to depend on them as a source of vitamin to any great extent.

Since the function of these food accessory substances has been appreciated, there has been a growing tendency on the part of those particularly interested in them to regard the fat of the diet of special importance only as a carrier of the fat soluble vitamin. These investigators seem inclined to attribute all the bad effects of a diet low in fat to vitamin deficiency. The fact that a diet deficient in fat predisposes to rickets, that it increases the susceptibility to infection and that it retards growth is explained by them to be a result of avitaminosis. The contention is that if sufficient fat soluble vitamin is supplied in a diet which is fat free, but otherwise adequate, the conditions just mentioned will not develop.

Most of the conclusions regarding the functions of vitamins have been drawn from experiments on small laboratory animals, chiefly rats. Mendel and Osborne[1] have recently published the results of

1. Osborne, T. B., and Mendel, L. B.: J. Biol. Chem. **45**:145 (Dec.) 1920.

*experiments in which they obtained normal growth in rats on a diet extremely low in fat, but which included the necessary amount of the fat soluble vitamin, supplied in their experiments by dried alfalfa leaves. They conclude that "if true fats are essential for nutrition during growth the minimum necessary must be exceedingly small."

To apply dogmatically to human beings the results of experiments on small animals—such as rats, which are of a species so different— seems open to question. The natural diet of the rat is very different from that of the human and consequently its digestive processes may not be entirely comparable. Hence, it is conceivable that fat may have functions in human nutrition, quite apart from that as a pur- veyor of vitamin A, which may not be required of it in the nutrition of the rat.

Von Gröer[2] has observed the effects of a diet practically fat free on two infants from birth. The food given was centrifugalized cow's milk containing only 0.01 per cent of fat with the addition of sufficient cane sugar to raise the carbohydrate content to about 8.5 per cent of the food mixture. This diet was not only practically devoid of fat, but also extremely low in fat soluble vitamin and high in carbohydrate. Both the infants observed gained in weight with fair regularity for six months, one at the rate of 15 gm. per day and the other at the rate of 20 gm. per day. After six months the gain ceased in both. Both developed rickets and were susceptible to infection throughout the period of observation. The conclusion reached by Von Gröer is that for at least the first six months of life infants can be nourished success- fully on a diet which is practically fat free, provided the calories needed are supplied by sugar. His results hardly bear out this contention.

Another function of fat which is questioned by some is its influence on mineral metabolism. This has been much debated and the sub- ject cannot be regarded as entirely settled. Many laboratory obser- vations have been made to ascertain the influence of the fat intake on the mineral metabolism of infants and very young children. Un- fortunately, the results thus far have been contradictory and definite conclusions have not yet been reached. This is probably due to the inadequacy of the data.

2. Von Gröer, F.: Biochem. Ztschr. 97:311, 1919.

Although some investigators are convinced that there is no relation between the fat intake and the mineral metabolism, we feel certain that in the case of calcium metabolism at least a very definite relation exists. The work on calcium metabolism done in this laboratory *indicates that proper calcium absorption does not usually take place* unless the fat in the diet bears a certain relation to the calcium and unless the intake of both is liberal. Our results indicate that the daily fat intake of children from 2 to 4 years of age should not be less than 3 gm. per kilo to insure proper absorption of calcium. This requirement seems to diminish up to 6 years of age to about 2 gm. per kilo, but is probably maintained at about this figure throughout the remainder of childhood. Since adequate absorption of calcium is necessary for bone growth, this need seems a very important one. Our results show that for the purpose of calcium absorption either animal or vegetable fat will serve equally well.

·Some authors have recently taken the ground that the fat of the diet has an important influence on the metabolism of protein. Maignon[3] has discussed this question rather fully. He believes that without a proper proportion of fat in the diet the protein is not economically utilized, and also that under these conditions protein forms toxic products which may be distinctly harmful. He considers that the amount of fat in the diet, should not be regarded as supplementary to the dietary requirement but an integral part of it. Maignon regards as significant the fact that both in cow's milk and in woman's milk the fat exists in greater amount than the protein.

Orgler[4] believes that, although the exact function of fat has as yet not been determined, the lack of fat certainly predisposes to edema and increases susceptibility to infection, especially to tuberculosis. This belief is quite generally accepted. Although children may apparently do well for a time on a low fat or a fat free diet, it is Orgler's opinion that a digestive upset almost inevitably follows. He considers that the complete elimination of fat from the diet or even its reduction to insignificant proportions, as proposed by Pirquet in connection with his new system of feeding, is a hazardous experiment.

3. Maignon, A.: Ann. de méd. 7:280, 1920; Compt. rend. Soc. de biol. 82:400, 1919.
4. Orgler, A.: Deutsch. med. Wchnschr. 46:290 (March 11) 1920.

It is unquestionable that a reasonable amount of fat in the diet is of service in maintaining normal conditions in the intestine. This is indicated by observations on the character and composition of stools. Stools which would be generally regarded as normal in character and consistency are not found when for a considerable time the fat of the diet has been excluded entirely or reduced to a very small amount. Only a small proportion—usually about 5 per cent—of the ingested fat is not absorbed, but this small amount has, we believe, as Hutchison[5] recently suggested, "a function to perform and is not a pure excretion." The fat excreted is largely in the form of soap, which gives solid consistency to the stools. Again, the reaction of the stool is to a great extent dependent on the relative proportions of fat and carbohydrate in the diet. When carbohydrate forms too large a proportion of the diet, the acidity of the stool is regularly increased. When, however, there is sufficient fat in the diet to supply a normal amount of soap in the stool there is no excessive acidity. Consequently, the fat in the diet may be regarded as exerting on the mucous membrane of the intestine a protective action against the irritating effects which may be produced by fermentation of carbohydrate when this element is given in excess. This will be considered more fully in discussing carbohydrate requirement.

Aside from these reasons for regarding fat per se as an important constituent of the diet of the child, it must not be forgotten that fat as a source of energy provides more than twice as many calories per gram as does either protein or carbohydrate.

From the foregoing it seems quite clear that a considerable amount of fat is desirable and probably essential for the best nutrition of the growing child. The exact quantity needed, however, is difficult to determine. At the present time it can only be conjectured. Many authorities think that the diet naturally chosen is an indication of a physiologic need.

Fat in the Diet of Infants.

That fat is a natural component of the diet of the infant is shown by the composition of woman's milk. The normal amount of fat in

5. Hutchison, H. S.: Quart. J. Med. 13:277 (April) 1920.

woman's milk, on the average between 3 and 4 per cent., furnishes approximately half the total calories in the diet of the nursing infant. This cannot be without great significance. It seems hardly conceivable that so much fat would be supplied by nature if the only specific purpose of the fat were to furnish the vitamin. The nursing infant usually receives during the early weeks about 20 gm. fat daily. This increases to an average of about 40 gm. daily at the sixth or seventh month. This represents about 4 gm. per kilo of body weight.

Nature provides fat generously not only for the nursing human infant but also for the young of all mammals. The various milks all contain a considerable proportion of fat; in many of them the fat occurs in greater proportion than in woman's milk. In most milks the amount of fat and protein run almost parallel, but in several the fat considerably exceeds the protein.

The infant fed on modifications of cow's milk does not usually receive as much fat as does the nursing infant. The complete digestion of the fat of cow's milk is difficult for many infants. Consequently, it must be given in smaller amounts and increased cautiously. A widely accepted basis for the artificial feeding of the average normal infant is an allowance of 1.5 ounces of cow's milk for each pound of body weight. The fat intake calculated on this basis ranges from 15 gm. daily during the early weeks to 35 gm. daily at one year. This provides about 3.5 gm. fat per kilo of body weight. The fat allowance for different infants varies rather widely according to the individual capacity to digest cow's milk fat. Many pediatrists are inclined to attribute most of the digestive disturbances of infants to the fat of the diet and to recommend the more extensive use of skimmed milk formulas. Others consider a more liberal intake of fat to be of great advantage to most healthy children and use whole milk or in certain cases top milk of varying degrees as the basis for the milk modifications.

Fat in the Diet of Older Children.

Although there are in the literature many statements regarding the amount of fat recommended for children after infancy, there are but few actual records of the amount of fat taken in individual cases and few observations as to the effects of either a very high or a very low

fat intake. The amount of fat taken by more than one hundred normal children studied by us may, perhaps, be regarded as typical of that usually taken by healthy children.

Total Fat Daily.—The total daily amount of fat which these children received in their diet is shown in Chart I. The lines represent the grams of fat daily which would supply 35 per cent. of the total calories recommended by us in a previous paper.[6] As will be shown later, healthy children take on the average about 35 per cent. of their

Average Fat Intake of Normal Children.

Age, years.	Boys.			Girls.			Both sexes.		
	No. of cases.	Fat intake, gm. daily.	Fat intake, gm. per kg.	No. of cases.	Fat intake, gm. daily.	Fat intake, gm. per kg.	No. of cases.	Fat intake. gm. daily.	Fat intake, gm. per kg.
1–2	2	36	3.7	5	36	3.2	7	36	3.3
2–3	4	42	3.1	7	53	3.9	11	49	3.6
3–4	6	44	3.2	4	64	4.1	10	52	3.6
4–5	7	57	3.3	5	58	3.2	12	57	3.3
5–6	5	63	3.2	5	59	3.4	10	61	3.3
6–7	4	78	3.4	4	55	2.8	8	66	3.1
7–8	2	88	3.5	3	73	2.9	5	79	3.1
8–9	4	92	3.4	5	82	2.9	9	86	3.1
9–10	5	101	3.5	4	70	2.6	9	87	3.1
10–11	6	99	3.2	3	68	2.0	9	89	2.8
11–12	3	106	2.7	1	81	2.2	4	100	2.6
12–13	1	118	2.6	2	87	1.9	3	97	2.1
13–14	0	...	...	1	83	1.6	1	83	1.6
14–15	2	152	2.7	1	70	1.5	3	124	2.4
15–16	2	142	2.4	1	117	2.2	3	134	2.3
16–17	1	121	2.5	0	...	...	1	121	2.5
17–18	0	...	...	1	101	1.8	1	101	1.8

total daily calories as fat. It will be seen that there is considerable variation in the individual values. However, there is a notable tendency to follow the lines indicated. Most of the instances of very high fat intake were in children of extreme activity. Two boys in their fifteenth and sixteenth years, respectively, took more than 150 gm. fat daily. This large amount is indicative of the needs and habits of adolescence.

6. Holt, L. E., and Fales, H. L.: Am. J. Dis. Child. 21:1 (Jan.) 1921.

In the accompanying table are shown the average values according to years for total fat intake of the cases studied. The average values are given for boys and girls separately as well as for both sexes together. There is a practically steady increase in the average intake, reaching a maximum during the fifteenth and sixteenth years, as would be expected. The boys show a rather higher average intake than the girls.

Fat Per Kilo.—The individual values for the grams of fat per kilo taken by these children are shown in Chart 2. Although there is a considerable variation in the values, more than three-fourths of the children took between 2 and 4 gm. per kilo daily. The amounts taken by the boys show less variation than do those by the girls. The girls with very high intake were all healthy but extremely active children. The chart shows that as age avanced there was a gradual reduction in the grams of fat taken per kilo.

The average values for grams of fat per kilo, shown in the table, are nearly the same for both sexes during the early years—a little more than 3 gm. per kilo. Between the ages of 6 and 11 the values for the boys average a little more than 3 gm. per kilo; those for girls are somewhat lower. After the age of 11 there are too few data to warrant conclusion.

Müller[7] found the average fat intake of thirty-two children from 2 to 6 years of age to be 3.45 gm. per kilo, both sexes being grouped together. This value agrees with our findings for corresponding ages. Most 'of the other observations reported by German authors show lower values. Those of Camerer, who has been widely accepted as an authority, are especially low, most of his values being less than 1.5 gm. per kilo. In his recommended schedule the fat allowance is only about one-half that which is usual in the diet of American children. Among the' authors who have proposed a scheme of feeding in which exact quantities of the food constituents are given, Steffen[8] is about the only one who recommends a generous fat intake. Pirquet[9] has taken the position that fat is not a fundamentally essential article of diet and that it can be largely, if not entirely, replaced by carbohydrate without harm to the organism.

7. Müller, E.: Biochem. Ztschr. 5:143, 1907.
8. Steffen, W.: Jahrb. f. Kinderh. 46:332, 1898.
9. Pirquet, C.: System der Ernährung, Berlin, 1917.

An important recent report on the amount of fat taken by older children is that found in the article by Gephart,[10] who studied the diet taken by the boys of St. Paul's School, to which we have referred previously. These boys took on the average about 200 gm. fat daily.
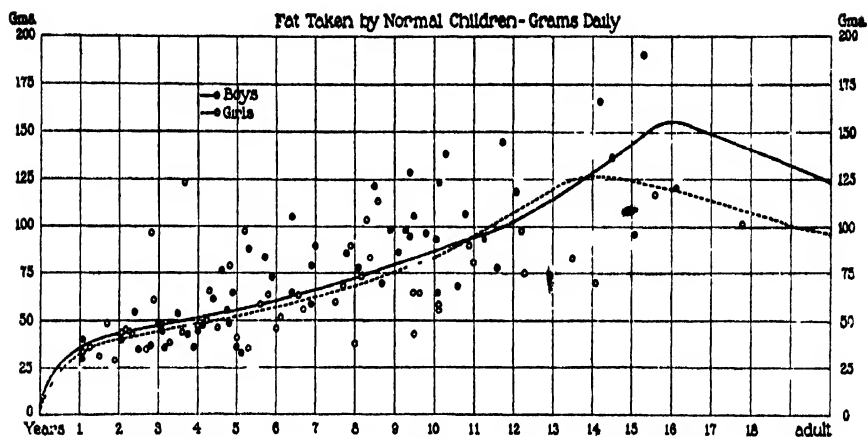
CHART 1
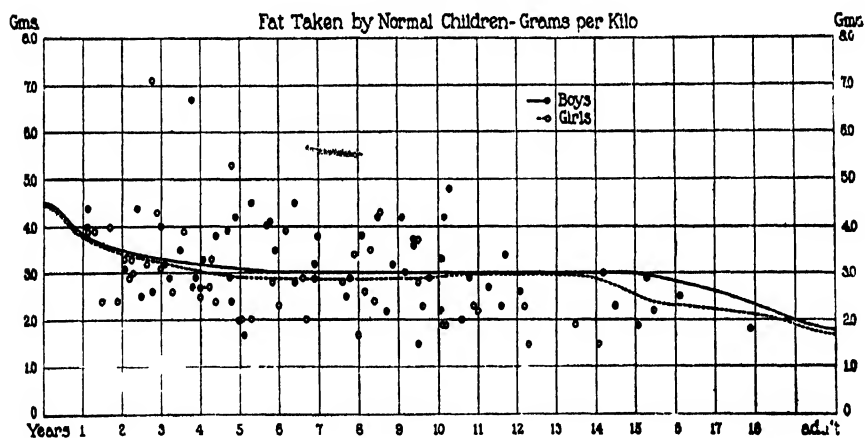
CHART 2

Of the three groups, the youngest, averaging 13½ years of age, took an average of 4.5 gm. fat per kilo; the middle group, averaging 14½ years, took 3.9 gm. fat per kilo; the older group, averaging 16¼ years, took 3.4 gm. fat per kilo. These values are much higher than has been thought desirable for those ages. The average adult diet, based on

10. Gephart, F. C.: Boston M. & S. J. 176:17, 1917.

many observations, contains only about one-half the amount of fat taken by these boys. Gephart's findings, however, are in accord with our belief that the food needs during adolescence considerably exceed those of adult life. The fact that the fat intake of so many healthy boys was so high is undoubtedly significant. This large amount of fat apparently had no deleterious effect; on the contrary, the boys thrived and it seemed to represent a physiologic need.

Certain clinicians are inclined to attribute many of the common digestive disturbances seen in older children to the fat in the diet, particularly the fat of cow's milk. It is undoubtedly true that under certain conditions a very high intake of fat, especially when accompanied by a great reduction in the carbohydrate intake, may bring about disturbances of digestion. The characteristic symptoms are coated tongue, bad breath, general malaise, constipation, with large gray stools containing a high proportion of fat, and sometimes severe and prolonged attacks of vomiting. In many such cases, however, when fat is blamed, the real cause of disturbance is too much food. This is apt to occur when children of limited activity are given more than a quart of milk, and that often rich milk, in addition to liberal amounts of solid food. It is important before lowering the fat intake to consider the diet as a whole, especially as to the total amount of food taken and the relative amounts of fat and carbohydrate which the diet contains. There is evidence that when a diet is properly balanced, that is, contains the fat, protein and carbohydrate in the right relation to each other, very much larger amounts of fat can be tolerated than when the diet is unbalanced.

Whenever the fat in the diet is much reduced it is necessary in order to supply sufficient calories to greatly increase the carbohydrate. When this is done there is always great danger of disturbing digestion from an excess of carbohydrate.

While it may not yet be established that a large amount of fat is essential in the diet during growth, there are so many reasons for a liberal allowance of fat and so little evidence that this is harmful to children with normal digestion that it does not seem wise with our present knowledge to reduce the fat much below the amount which healthy children usually take, while to exclude it entirely from the diet seems quite unjustifiable and hazardous.

SUMMARY.

1. Many of the functions of fat in the diet are still subjects of debate.

2. A certain amount of fat should be supplied to provide fat soluble vitamin. This amount is not yet known. It may be exceedingly small, but until this is known it seems wise not to reduce the fat supply greatly lest there may be a deficiency in vitamin A.

3. Fat probably has an important influence on mineral metabolism, especially on calcium metabolism. For the best absorption of calcium we have found it desirable to supply as much as 3 gm. fat per kilo in the early years and as much as 2 gm. per kilo after 6 years.

4. Fat is probably necessary for proper digestion and utilization of protein and on this account it seems desirable to supply as much fat as protein in the diet.

5. Fat helps to maintain normal physical, bacteriologic and chemical conditions in the intestine. It also exerts a protective action against the irritating effects of the products of carbohydrate fermentation.

6. A great reduction of fat in the diet increases the susceptibility to infection, especially to tuberculosis.

7. The nursing infant receives a generous amount of fat, usually as much as 4 gm. per kilo daily.

8. The infant taking modification of cow's milk usually receives about 3.5 gm. fat per kilo daily.

9. Older children studied by us took on the average over 3 gm. fat per kilo daily when under 6 years of age and about 3 gm. per kilo during the remainder of the growth period.

10. The grounds for the current impression regarding the harmful effects of fat on children should be investigated carefully. There is little evidence that a liberal amount of fat in the diet is harmful to children with normal digestion and much evidence that fat is an important and necessary component of the diet during the entire growth period.

11. In general, it seems rational to supply in the diet of the child as much as 4 gm. fat per kilo daily at one year, decreasing the amount to about 3 gm. per kilo at 6 years and maintaining this value throughout the remainder of the growth period.

STUDIES OF ACIDOSIS.

XVIII. Determination of the Bicarbonate Concentration of the Blood and Plasma.

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In order to determine the acid-base balance of the blood as outlined previously (Van Slyke, 1921) it is desirable to ascertain the bicarbonate concentration existing in the blood in the body. As pointed out in the above mentioned paper,[1] determinations of the CO_2 content of the whole blood (Peters and Barr, 1920–21) or plasma (Van Slyke and Cullen, 1917), after equilibration at an arbitrarily chosen CO_2 tension, or determinations of the plasma bicarbonate by titration to a constant pH as end-point (Van Slyke, Stillman, and Cullen, 1919), although adequate to indicate alkali deficit or excess, are not sufficient to indicate conditions in which the respiratory control of the CO_2 tension also is disturbed, and in consequence the blood pH is varied from the normal.

To determine the bicarbonate of the blood under the conditions existing *in vivo* we may proceed in either of two ways. We may determine the total CO_2 ($BHCO_3 + H_2CO_3$) of either blood or plasma, together with the pH, and estimate the $BHCO_3$ by the equation,

$$BHCO_3 = K'\frac{H_2CO_3}{[H^+]};$$ or we may titrate the plasma as described by

Van Slyke, Stillman and Cullen (1919), with the modification that the end-point of the titration is the pH of the individual plasma as drawn, rather than the constant pH 7.4 used by the above authors.

Gasometric Determination of Plasma of Whole Blood Bicarbonate.

The total CO_2 is determined in either plasma or whole blood (Van Slyke and Stadie, 1921). The plasma pH is determined either

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[1] Van Slyke (1921), pp. 171 and 172.

electrometrically or by the colorimetric method of Cullen (1922). The bicarbonate is then calculated by the factor given in Table I. The factors in Table I are calculated from the mass action equation,

$$[H^+] = K' \frac{H_2CO_3}{BHCO_3},$$ transformed as follows. Expressing the total

CO_2 concentration as $[CO_2]$, the free dissolved CO_2 as $[H_2CO_3]$, the bicarbonate as $[BHCO_3]$, we have Equation 1, which is L. J. Henderson's expression of the mass law as applied to $BHCO_3 + H_2CO_3$ solu-

TABLE I.

pH	Total CO₂ in the form of bicarbonate.	
	Whole blood.	Plasma or serum.
	per cent	*per cent*
7.0	87.6	88.5
7.1	89.9	90.9
7.2	91.8	92.7
7.3	93.4	94.1
7.4	94.8	95.3
7.5	95.8	96.2
7.6	96.6	97.0
7.7	97.3	97.6
7.8	97.8	98.1

tions (1909). Equations 2 and 3 are the intermediate steps leading to Equation 4. pK' is the negative logarithm of K', as pH is of H⁺.

(1) $$[BHCO_3] = K' \frac{[H_2CO_3]}{[H^+]}$$

(2) $$[BHCO_3] = K' \frac{[CO_2] - [BHCO_3]}{[H^+]}$$

(3) $$[BHCO_3] = \frac{1}{1 + \frac{[H^+]}{K'}} [CO_2]$$

(4) $$[BHCO_3] = \frac{1}{1 + 10^{pK' - pH}} [CO_2]$$

$\frac{1}{1 + 10^{pK' - pH}}$ in Equation 4 is the fraction of the total CO_2 in the form of bicarbonate.

The value of pK' was originally determined by Hasselbalch (1917) to be 6.03 for 0.05 M NaHCO$_3$, 6.06 for 0.03 M, and 6.08 for 0.02 M[2]. In whole blood Hasselbalch found that the same values held. Haggard and Henderson (1919) from a compilation of the data in the literature estimated the average value of K' for whole blood to be 8×10^{-8} whence pK' = 6.10. In work shortly to be reported from this laboratory the value of pK' has been found to be 6.15 for normal whole blood, 6.10 for plasma. From these values of pK' the values of $\dfrac{100}{1 + 10^{pK'-pH}}$, the percentage of total CO$_2$ in the form of bicarbonate, given in Table I are calculated.

Titration of Plasma Bicarbonate.

This determination may be conveniently combined with the colorimetric method of Cullen (1922), since the diluted 1 cc. sample of plasma used in Cullen's pH determination serves to indicate the end-point in the bicarbonate titration. The oxalated blood is drawn and centrifuged under rigorous precautions to avoid loss of CO$_2$ which are detailed by Cullen (1922) in the accompanying description of his colorimetric pH determination. 1 cc. of the plasma, to serve as a standard for the end-point, is treated exactly as described by Cullen to prepare it for colorimetric pH determination, by diluting it under oil, in a tube of 20 mm. diameter, with 20 cc. of neutral 0.9 per cent NaCl solution, containing 7 drops of 0.03 per cent phenol red.

Another 1 cc. sample of the plasma is transferred to a round flask of about 100 cc. capacity, 5 cc. of 0.01 N HCl, which is made up in neutral 0.9 per cent NaCl, are added, and the CO$_2$ is removed by whirling the mixture about the flask for at least 1 minute, as described by Van Slyke, Stillman, and Cullen (1919). The solution is poured into a test-tube of the same diameter as that containing the standard, the portion adherent to the walls of the flask being transferred by rinsing with 10 cc. of 0.9 per cent NaCl divided into three portions.

[2] The figures actually given by Hasselbalch for pK' are 0.3 higher, because he used the equivalent concentration of H$_2$CO$_3$, which is twice the molecular, in calculating the BHCO$_3$: H$_2$CO$_3$ ratio. When the molecular ratio employed by most other authors is used, Hasselbalch's data give the figures quoted.

7 drops of the 0.03 per cent phenol red solution are added, and 0.01 N NaOH is run in from a burette, which permits readings to 0.01 cc., until the color matches that of the standard. As the end-point is approached, sufficient 0.9 per cent NaCl is added to bring the volume to 20 cc.

The 0.01 N NaOH like the 0.01 N NaCl, is made up by diluting 1 volume of 0.1 N pure solution to 10 volumes with neutral CO_2-free 1 per cent NaCl.

TABLE II.

Plasma No.	Titration method.		Gasometric method.				
	0 01 N HCl used in titrations.	BHCO₃ concentration.	Total CO₂.	pH by Cullen's method.	Proportion of total CO₂ as BHCO₃ (from Table I).	BHCO₃	
	cc.	*milli-molecular*	*vol. per cent*		*per cent*	*vol. per cent CO₂*	*milli-molecular*
1	2.14						
	2.15	21.5	51.6	7.44	95 7	49.3	22.0
2	1.61						
	1.61	16.1	37.6	7.44	95.7	36.0	16.1
3	1.30						
	1.26	12.8	30.9	7.38	95.1	29.4	13.1
4	1.22						
	1.24	12.3	30.3	7.22	92.8	28.1	12.5

The use of saline solution instead of water has the advantage of preventing the formation of a permanent cloudy precipitate of globulin.

The precautions outlined by Van Slyke, Stillman, and Cullen (1919) and by Stillman (1919), in particular those concerning the avoidance of CO_2 in the standard NaOH solution, are to be observed.

The nature of the results obtained is indicated by Table II.

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STUDIES OF ACIDOSIS.

XIX. The Colorimetric Determination of the Hydrogen Ion Concentration of Blood Plasma.*

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The electrometric method for determining the blood reaction requires relatively large quantities of blood, elaborate apparatus, constant attention, and extremely careful manipulation when dealing with CO_2-containing solutions, and is, therefore, of only limited availability. The colorimetric methods so far reported have not been comparable in accuracy to the electrometric methods.

Levy, Rowntree, and Marriott (1915), added phenol red to the dialysate of the blood and matched the color produced against the color standards, thus avoiding the difficulties caused by the color of the blood as well as by the protein effect of the plasma proteins upon the dye. Although, because of several inherent errors, the method is not suited for exact determination of the actual reaction of the blood, the method has been of great value in affording a measure of the change in reaction.

The more important errors in this method were: (1) loss of carbon dioxide; and (2) the difference in the reaction of two solutions, one containing protein, when separated by a semipermeable membrane. This phenomenon, the Donnan "membrane equilibrium," has been recently studied by Loeb (1921–22). Minor errors involved were the variable dilutions and temperature errors.

Attempts to prevent the loss of CO_2 have been reported by Scott (1917) and more recently by Dale and Evans (1920–21). Dale and Evans enclosed their dialysis membrane in a CO_2-tight container.

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* A preliminary report of this work appeared in the Proceedings of the American Society of Biological Chemists (*J. Biol. Chem.*, 1922, 1, p. xvii).

They used neutral red as an indicator, which was added to the dialysate after the dialyzing membrane had been removed and before the liquid paraffin had been added.

The technique for colorimetric pH determination has been greatly refined in recent years, especially by Walpole's (1914) introduction of the comparator and by Clark's (1920) studies, and it was felt that it should now be possible to develop an accurate colorimetric method for the determination of the pH of blood plasma. (Parsons (1919–20) has recently shown that when whole blood is used for the electrometric determination, the pH obtained is that of the plasma, the suspended corpuscles not affecting the result.) In an accompanying paper Cullen and Hastings (1922) show (contrary to the finding of Evans), that when proper precautions are observed, the colorimetric agrees exactly with the electrometric method in CO_2-containing solutions as well as in phosphate solutions.

The method which has been developed and which will be described below eliminates the Donnan effect by determining the reaction of the diluted plasma itself, and allows for the protein, salt, dilution, and temperature effects, by a correction experimentally determined by comparison of colorimetric results with those by the standard electrometric method.

EXPERIMENTAL.

Dilution Error.—As is evident from Fig. 1, serum showed a continued increase in alkalinity when diluted with 0.9 per cent NaCl solution up to a dilution of 15- to 20-fold. Beyond 20-fold the change upon further dilution is so small as to be practically negligible. A dilution of 20-fold with 0.9 per cent saline solution was therefore chosen as optimal. A dilution as great as this has the further advantage of making it possible to work with as little as 0.5 to 0.25 cc. of serum, obtainable from 1 cc. of blood.

The use of salt solution instead of water as a plasma diluent is necessary because the curve is not only steeper with water than with saline solution, but also because with water globulin precipitation occurs before the plateau of the curve is reached.

Addition of neutral potassium oxalate to the blood up to 1 per cent does not effect the dilution curve.

Temperature Effect.—The effect of change in room temperature upon the colorimetric reading is shown in Fig. 2. This curve is iden-
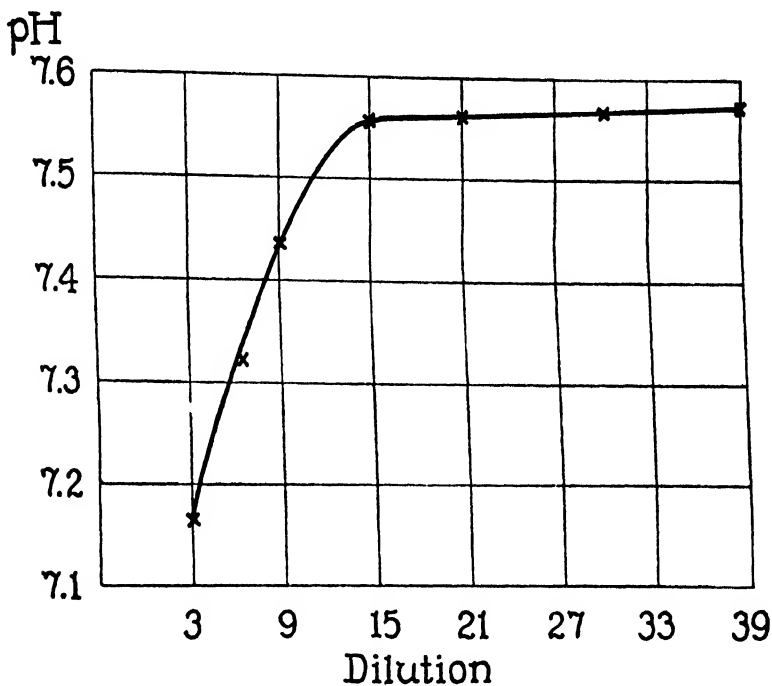
FIG. 1.
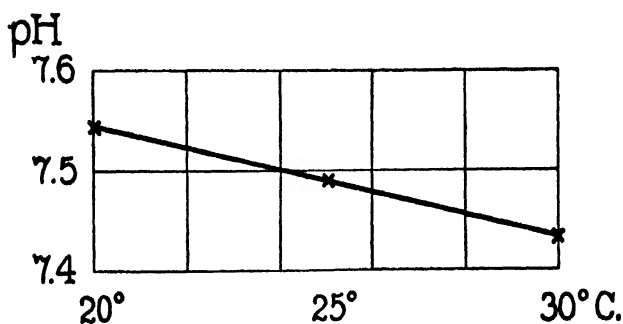
FIG. 2.

tical with several obtained in dilute plasma solutions. Both the unknown and the phosphate standards containing phenol red were at the temperature indicated

The temperature correction is $+ 0.01$ pH per increase of $1°C$.

Hemolysis.—Slight hemolysis does not appear to interfere appreciably with the reading, but with any marked hemolysis the change in

TABLE I.

Comparison of Colorimetric (20°) and Electrometric (38°) pH Values of Horse Plasma.

No.	Total CO₂ content.	CO₂ tension.	Colorimetric 20°C.	Electrometric 38°C.	Colorimetric at 20° minus electrometric at 38°	Deviation from average difference.
	millimols	*mm. Hg*	*pH*	*pH*	*pH*	*pH*
1	28.0		7.57	7.42	0.15	+0.03
2	27.7	49.5	7.52	7.39	0.13	+0.01
3	28.5		7.53	7.41	0.12	0.00
4	27.2	44.7	7.53	7.38	0.15	+0.03
5	26.7		7.55	7.43	0.12	0.00
6	27.1	44.5	7.54	7.42	0.12	0.00
7	27.9		7.50	7.42	0.08	−0.04
8	28.0	20.0	7.73	7.63	0.10	−0.02
9	25.9	38.7	7.57	7.45	0.12	0.00
10	29.3	57.9	7.45	7.33	0.12	0.00
11	18.1	19.2	7.72	7.57	0.15	+0.03
12	24.5	45.6	7.48	7.35	0.13	+0.01
13	20.3	19.4	7.69	7.63	0.06	−0.06
14	26.7	45.6	7.51	7.40	0.11	−0.01
15	27.7	47.3	7.51	7.40	0.11	−0.01
Average for plasma................................					0.12	±0.02

The values in Tables I and II represent the electrometric reading at 38°C. on undiluted plasmas and sera of bloods equilibrated at 38° at the indicated CO₂ tensions as compared with the colorimetric readings corrected to 20°C. of 20-fold dilution with saline solution of the same plasma or serum. Where no CO₂ tension is given the determination was carried out on the plasma of the blood as drawn. The total CO₂ content is given to show there is no relationship between the CO₂ content and the deviation figure.

the tone of the dye is so great that accurate readings are impossible. Since serum may be used instead of plasma, oxalate may be dispensed with when it causes hemolysis.

Total Correction.—Since the primary interest in the reaction of the blood is in the reaction at body temperature it was decided to refer all colorimetric readings made at room temperature to the electrometric value at 38°. Simultaneous determinations, electrometric at 38° on undiluted plasma and colorimetric at room temperature on 20-fold dilute plasma, were carried out on a series of bloods which had previously been equilibrated with gas mixtures of varying CO_2 tension. The results of twenty-three such determinations on horse blood are given in Tables I and II. The colorimetric determinations are all corrected to 20°C., *i.e.*, $pH_{20°} = pH_{t°} + 0.01\ (t° - 20°)$ where $t° =$ room temperature.

TABLE II.

Comparison of Colorimetric (20°) and Electrometric (38°) pH Values of Horse Serum.

No.	Total CO_2 content.	CO_2 tension.	Colorimetric 20°.	Electrometric 38°.	Colorimetric at 20° minus electrometric at 38°.	Deviation from average difference.
	millimols	*mm. Hg*	*pH*	*pH*	*pH*	*pH*
1	29.96		7.60	7.46	0.14	−0.02
2	22.1	17.9	7.81	7.65	0.16	0.00
3	27.51	38.8	7.65	7.47	0.18	+0.02
4	31.28	57.9	7.50	7.36	0.14	−0.02
5	29.9		7.58	7.45	0.13	−0.03
6	28.4	45.6	7.58	7.41	0.17	+0.01
7	28.7	45.6	7.59	7.43	0.16	0.00
8	28.8	45.6	7.59	7.43	0.16	0.00
Average for serum..					0.16	±0.02

It is apparent that there is a distinct difference between the correction for plasma and that for serum. The same difference was found in another series of twenty-two sera and fourteen plasmas. These determinations were made on the same horse and it is felt that the difference between the plasma and serum correction must be due to the presence of the fibrin in the plasma, since the addition of neutral potassium oxalate to defibrinated blood or to the separated plasma or serum in concentration up to 1 per cent does not affect either the electrometric reading or the colorimetric reading on the diluted serum or plasma. Moreover, the same difference is noted when the plasma

and serum are obtained from different portions of the same sample of blood where they have the same actual (electrometric) pH. The constancy, however, of the correction value for the fifteen plasmas at 0.12 pH and of the eight sera at 0.16 pH is striking.

In addition to the difference between the plasma and serum of horse blood, it has been found that the same fluid (*e.g.*, plasma) has different correction values in different species. In Table III the extent of this variation is indicated. It is possible also that in some pathological conditions the difference between the electrometric and colorimetric pH reading may be altered. This question is being studied

TABLE III.

Average Values of Correction for Different Systems.

Number of specimens in series.	System.	Average correction.	Average deviation.	Maximum deviation.
10	Human plasma.	0.23	0.02	0.04
5*	Human plasma.	0.22	0.00	0.01
7*	Rabbit plasma.	0.17	0.01	0.02
4	Dog serum.	0.35		0.03
9†	Human joint fluid.	0.21	0.026	0.05

* These five human plasma values and seven rabbit plasma values were furnished by Dr. A. B. Hastings (personal communication).

† Boots and Cullen (1922).

further. The data given for the correction for colorimetric pH readings in fluids other than human plasma are too few in number to establish reliable values for the correction in these instances, but serve to indicate its nature.

However, the seventeen values for human plasma, which were obtained from seventeen individuals, indicate that the value 0.22 is accurate to within the combined error 0.04 pH of the two methods.

Calculation.—The calculation of the reaction of the blood at 38° is as follows: $pH_{38°} = pH_{colorimetric 20°} - C$, where C is the appropriate correction for the system under investigation. If it is not

feasible to make the determination at 20°C. the colorimetric reading for horse or human blood may be corrected as indicated above.

$$\text{Colorimetric pH }_{20°} = \text{colorimetric pH }_{t°} + 0.01 \; (t° - 20°).$$

Although this temperature correction appears accurate for the range 20–30°, because of the empirical nature of the correction, it is desirable to make all determinations at 20°. This is especially true in using the method with other than horse or human blood. For values reported above, the room temperatures were used either at 20° or between 20 and 24°.

Application of the Method.—The reliability of the colorimetric method in its present state of development should be considered from two distinct view-points.

1. Determination of Relative pH Changes.—In studying the changes in reaction which may occur during the course of an experiment or in following the condition of a patient, for example, under alkali therapy, the proposed method can be used with an accuracy of at least ± 0.04 pH and probably with an accuracy of ± 0.02. Whether or not extreme pathological conditions change the correction remains to be determined.

2. Determination of Absolute pH Values.—The variations in the correction factors which are given in Table III (from 0.11 pH for horse plasma to 0.35 pH for dog serum) make it necessary to determine the correction factor for each particular species. However, the individual variations from the average corrections in the cases of human and horse plasma and horse serum are so small as to make it appear probable that the species corrections determined are approximately constant, and that the method can be used with an accuracy of about pH ± 0.04. The method has already proved of clinical value in detecting gross deviations (0.2 to 0.3 pH) from the normal in conditions such as nephritic acidosis.

Details of Determination.

Blood Sampling.—The blood is drawn without stasis and without exposure to air into a glass syringe or tube coated with potassium oxalate to make 0.3 per cent and containing mineral oil. (Mineral oil, potassium oxalate, and glassware must be tested for neutrality.)

Then, without exposure to the air, the blood is run into a tube under oil to the complete filling of the tube. A one-hole rubber stopper is slipped into the tube, expelling through the hole the oil that remains over the blood. The hole is closed with a glass plug, the tube is placed in a centrifuge, and whirled. The plug is then taken out and as the stopper is removed from the tube, oil is allowed to run in through the hole in the stopper to cover the surface of the plasma so that it is never exposed to air. The plasma is then transferred under oil to another tube. Simply covering the blood with oil is not sufficient to prevent the loss of CO_2 during centrifuging. Oil suffices to prevent loss of CO_2 from solutions which are standing quietly for short periods only.

Determination.—35 drops of 0.03 per cent phenol red solution are added to 100 cc. of 0.9 per cent sodium chloride solution, freshly prepared from redistilled water. 1 to 3 drops of 0.02 N NaOH are added to bring this solution to pH 7.4 or 7.5. 20 cc. portions are then placed in suitable tubes (see below) and covered with mineral oil. Other tubes are prepared with 20 cc. saline solution without indicator.

A 1 cc. portion of the plasma is then allowed to run under the oil into the indicator-saline solution and another 1 cc. portion into the 20 cc. of saline solution. This latter tube is for use with the pH standard in the comparator. A 1 cc. bulb pipette, graduated to deliver between two marks, is convenient as it is otherwise difficult to deliver all the plasma from the tip of a pipette which is under a layer of oil. The plasma and saline solution are then mixed by introducing a stirring rod through the oil, and the pH determination is made by placing the tubes in a comparator block, and matching to the nearest standard color tube. It is possible to read to 0.01 or 0.02 pH. The temperature of the solution is determined by inserting a thermometer into the solution immediately after the pH reading. The reading should, if possible, be made at 20°. This can be conveniently accomplished by placing the diluted plasma tubes together with the necessary standard tubes in a large beaker of water at 20°. When they have attained 20° they are removed, and placed in the comparator. The reading is easily made before significant temperature change occurs. The pH observed is corrected to 38° by the formula given above.

Indicator Solution.—5 drops of 0.03 per cent phenol red solution are added to 15 cc. of the standard solution. The concentration of dye required varies somewhat with different lots. It is best to prepare a concentrated stock solution, and determine by experiment the dilution required to give satisfactory depth of color over the desired pH range. The indicator solution must be neutral. After the addition of 1 drop to 3 cc. of redistilled water, the water must not be red.

Standards.—Sörensen's phosphate standards are prepared from Merck's special reagents in steps of pH 0.05 from about pH 7.2 to 7.7. The M/15 phosphate solution should be prepared from special reagent salts (Merck's are satisfactory) by dissolving the following quantities to a liter with distilled water:

gm.
Na$_2$HPO$_4$ · 2 H$_2$O "Sörensen salt" 11.87
Na$_2$HPO$_4$ anhydrous (Merck).. 9.47
KH$_2$PO$_4$.. 9.08

The proportions of acid and alkaline phosphates are given in Table IV. These mixtures may be kept for some weeks in Pyrex glass in the refrigerator.

TABLE IV.

Phosphate Mixtures (Phenol Red Range).

pH	M/15 Na$_2$HPO$_4$	M/15 KH$_2$PO$_4$
	cc.	cc.
7.0	61.1	38.9
7.05	63.9	36.1
7.10	66.6	33.4
7.15	69.2	30.8
7.20	72.0	28.0
7.25	74.4	25.6
7.30	76.8	23.2
7.35	78.9	21.1
7.40	80.8	19.2
7.45	82.5	17.5
7.50	84.1	15.9
7.55	85.7	14.3
7.60	87.0	13.0
7.65	88.2	11.8
7.70	89.4	10.6
7.75	90.5	9.5
7.80	91.5	8.5

The color standards contain phenol red and must be renewed or checked against a fresh tube of dye at least once every week; as there is a slow fading of color.

Apparatus.—The comparator required is conveniently made from a block 3 × 6 inches. The holes for the tubes are of 1 inch diameter. The slits for the light are best made by boring two ½ inch holes and gouging out the remaining wood with a chisel. The tubes must be of clear, non-alkaline glass of *uniform* diameter. Tubes 20 mm. in diameter are convenient.

Light.—Either daylight or "Daylite" lamps are satisfactory.

Test for Neutrality.—The redistilled water is usually about pH 6.2 to 6.5. The easiest test is that of using both phenol red and methyl red. The water should give no (red) color with either indicator.

The syringe, pipettes, and tubes should be rinsed with redistilled water and dried. Syringes, tubes, and pipettes, washed and sterilized in the usual manner prevalent in bacteriological laboratories, are often dried from alkaline water.

The saline solution must be adjusted to pH 7.4 as described above.

The oxalate, when dissolved in water to a 0.5 per cent solution, should not be more alkaline than pH 7.2 to 7.4. The oil is tested by shaking with water containing phenol red and methyl red. The water must remain neutral.

Methods.

CO₂ Equilibration.—About 50 cc. of the blood to be equilibrated were placed in an 800 cc. tonometer. The tonometer was partially evacuated and the calculated amount of CO_2 was then added from a burette. The tonometer was then equilibrated in a bath at 38°. The formula used for the calculation where the tonometer is not equilibrated to atmosphere after the attainment of the temperature (*i.e.* constant volume) is

$$cc._{CO_2} = \frac{p_{38}{\cdot}CO_2 \times T_{burette} \times (V - s)}{311 \times (B - w)}$$

where p_{CO_2} = tension CO_2 in mm.
T = absolute temperature.
V = volume of tonometer.
s = volume of solution.
B = barometric pressure.
w = vapor pressure.

The details of technique and the gas manifold used are described in detail elsewhere (Austin, Cullen, Hastings, McLean, Peters and Van Slyke). After equilibration, without loss of CO_2, the blood was transferred to a second tonometer and again equilibrated to the same CO_2 tension. The solution was then transferred to a sampling tube over mercury from which samples were removed for colorimetric and electrometric determinations.

Colorimetric Determination.—The colorimetric readings were made in clear glass tubes of uniform bore (20 mm.). Redistilled water was used in making the 0.9 per cent saline solution and only fresh solutions were used. (The amount of CO_2 taken up by the saline solution upon standing may be sufficient to affect the reading.) An absolute guarantee of neutrality is conveniently attained by adding the 35 drops of dye to 100 cc. of saline solution and adjusting with 1 or 2 drops of 0.02 M NaOH to a ph of about 7.4 before measuring the 20 cc. portion into the tubes.

The readings were carried out in a comparator block using either daylight or the "Daylite" glass of the Nela Electric Co. Sunlight must be avoided. If turbid the plasma tubes should be placed alongside each other and next to the light.

All the measurements reported above were made in duplicate tubes. On each duplicate two or three readings were taken by each of two observers. It has been found desirable to make two or three rapid readings rather than strain the eyes by one prolonged comparison. The two observers always agreed within 0.01 pH.

The temperature of the colorimetric reading was obtained by placing a thermometer in the solution immediately after the reading.

The proportions of acid and alkaline phosphate given above were obtained from a large curve constructed by the use of a flexible spline from Sörensen's corrected figures. The color standards fade during the course of a week from 0.02 to 0.04 pH and it has been found convenient to make a quick comparison against the old standards and then to add dye to the fresh portion of standards with pH value on either side of the trial reading. This takes only a moment since the phosphate without dye keeps for a considerable period.

Electrometric Measurement.—The determinations were carried out in a constant temperature room at 20° or in a thermostat maintained at 38°. Clark electrode vessels of 2 cc. capacity and saturated calomel electrodes with a saturated KCl bridge were used. For plasma determination a mixture of hydrogen and CO_2 at the same CO_2 tension at 38° as that used in equilibration was run into the electrode chamber which had previously been washed with water and filled with hydrogen. The CO_2 tension was calculated on the basis of expansion under atmospheric pressure since the readings were made with the cocks open so that the hydrogen was at atmospheric pressure. The formula used was

$$cc.\, CO_2 = \frac{pCO_2 \times V}{(B - w)}$$

pCO_2 = tension CO_2 desired at 38°.
w = vapor pressure at 38°.
V = volume of tonometer.
B = barometric pressure.

After the initial reading, the plasma in the cell was replaced with another portion of plasma under the same bubble of hydrogen + CO_2 in the manner used by Hasselbalch to bring the hydrogen atmosphere to the same CO_2 tension as the solution. If the second reading checked with the first to within 1 millivolt, it was used as the correct reading. If it did not agree with the first refilling, the refilling and determination were repeated.

Our results agree with those of Hasselbalch in that, even when the electrode contained pure hydrogen at the start, only one or two refillings of blood are necessary to attain equilibration. The buffer value of plasma, however, is so low that if pure hydrogen is used five or six more refillings are necessary. It is possible, however, to get satisfactory results when the CO_2 tension is not known if the CO_2 tension in the hydrogen is approximately that of the plasma. For normal blood this may be taken as 40 mm. of Hg. Two or three refillings will then give constant readings. The measurements reported above, used in establishing the correction factors for the colorimetric method, are all made upon solutions equilibrated at known tensions.

Standardization.—For reasons which will be discussed more fully in a subsequent note by Cullen and Hastings the electrometric deter-

minations were standardized against 0.1 N HCl prepared by the method of Hulett and Bonner (1909) from constant boiling acid. We have assigned to it the values, derived from Noyes and Ellis (1917) for the activity coefficient, namely pH = 1.085 at 20° and pH = 1.090 at 38°, and have assumed that the temperature change of the activity coefficient is a linear function of the temperature. We have used the saturated calomel electrode without correction for diffusion potential (See Fales and Mudge, 1920).

Our procedure was to determine the "e" $\left(pH = \dfrac{\text{E.M.F.} - \text{"e"}}{0.0001984\ T} \right)$ of the entire system, including calomel cell and platinum electrode against 0.1 N HCl. One system was maintained at 38°, another at 20°. Using the values for "e" thus obtained—phosphate mixtures "7.4" were standardized against the 0.1 N HCl. This standard phosphate solution was then used before and after each set of plasma determinations to reëstablish the "e" of the system. If the "e" changed during a determination that determination was rejected. By this technique the danger of the slow liberation of acid absorbed by the platinum black is minimized. The phosphate solution was, of course, removed from the cell by thorough washing with distilled water.

At 20° the pH of the phosphate solutions standardized in this way against 0.1 N HCl (assuming pH 1.085) differ according to our experience, by about 0.01 pH from the values assigned to the mixtures by Sörensen. It is felt that this difference is a satisfactory agreement with Sörensen's values.

Platinum Electrodes.—Platinum electrodes were plated from platinum chloride solution containing 1 to 2 per cent HCl. Lead acetate was *not* used. After a satisfactory coating was obtained, the electrodes were placed in strong dichromate sulfuric acid clearing solution and heated over the water bath. The electrodes were then washed and allowed to stand in distilled water over night. This technique was proposed by J. C. Baker and L. L. Van Slyke (1918). Electrodes prepared in this manner may ordinarily be used for a long while, but when used with plasma the only safe procedure is to use fresh electrodes each day for four to six determinations on any one day. Something happens to the electrode used in such protein solutions, probably fibrin precipitation, which makes them unreliable for further

use in protein solutions. Electrodes, which will not give consistent, consecutive, or duplicate results with plasma, will appear perfect when rechecked against standard phosphates. Used again with plasma, no satisfactory reading can be obtained.

This undeterminable error which cannot be detected by the use of standard solutions can be recognized by the disagreement between duplicate measurements or between successive refills. For this reason plasma determinations were always duplicated simultaneously in two separate electrode vessels. The platinum electrodes after use with plasma may be cleaned with cleaning mixture, as outlined, without recoating. Our experience has been that platinum electrodes coated with platinum black are more satisfactory than those coated with palladium.

Thermostat.—The 38°C. measurements were carried out in a gas chain mounted in an incubator equipped with 150 watt nitrogen lamps for heating. The temperature was controlled with a De Khotinsky regulator and the air was stirred vigorously by means of a small fan. The temperature was read from a thermometer placed along side the Clark vessel. 10 minutes were required before the temperature of the solution agreed with that of the bath. This was determined by inserting a thermometer into the Clark cell in the manner described elsewhere (Cullen, 1922). Determinations at 20° were carried out in a constant temperature room.

Corrections.—The readings, made with a Leeds and Northrup potentiometer and wall galvanometer, were read to 0.2 millivolts and were then corrected for barometric pressure, vapor pressure, and CO_2 tension to 1 atmosphere of dry hydrogen at the temperature of the determination. No corrections were made for liquid junction potentials.

I am indebted to my assistant Mr. Julius Sendroy for his interest and coöperation during the development of this method.

SUMMARY.

1. A method for the colorimetric determination of the reaction of the blood is described. It is based upon the experimentally determined factor required to convert the colorimetic reading of diluted plasma or serum at room temperature to the actual reaction of the

plasma undiluted at 38° as determined electrometrically. This factor is somewhat different for plasma and for serum and for bloods of different species, but appears to have a constant value for each system.

2. A convenient and accurate technique for electrometric pH measurements at definite CO_2 tensions is outlined.

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A COMPARISON OF COLORIMETRIC AND ELECTRO-METRIC DETERMINATIONS OF HYDROGEN ION CONCENTRATIONS IN SOLUTIONS CONTAINING CARBON DIOXIDE.

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In connection with the development of a colorimetric method for measuring the reaction of the blood it became important to determine the precision of the colorimetric method as compared with the electrometric method when used with solutions containing carbon dioxide.

Experiments were therefore carried out on solutions of sodium bicarbonate which had been equilibrated with known tensions of carbon dioxide and in a similar manner on solutions of sodium bicarbonate plus sodium phosphate. The colorimetric readings were made under paraffin oil in the manner described in the preceding paper,[1] except that the solutions were not diluted. Each determination reported here is the average of duplicate readings by either two or three observers. Phenol red was used in preference to neutral red because of the marked tendency of neutral red to precipitate out of solution. Sörensen's phosphate standards[2] were prepared at intervals of 0.05 pH and the readings were made to 0.01 pH.

The equilibrations of the carbonate and carbonate-phosphate solutions with CO_2 were carried out in 800 cc. tonometers. The CO_2 tensions were prepared by running the calculated amounts of CO_2 from a burette into partially evacuated tonometers. The solutions were equilibrated in two or three successive tonometers and were then transferred, without exposure to air, to sampling tubes filled with mercury. (The details of the technique will be described in a paper appearing in a later number of this Journal by Austin, Cullen, Hastings,

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[1] Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

[2] Sörensen, S. P. L., *Ergerbn. Physiol.*, 1912, xii, 393.

McLean, Peters, and Van Slyke.)   Samples were then removed for determination.   In most experiments equilibrations were carried out in a 20° water bath.   The analyses were made in a 20° constant tem-

TABLE I.

Experiment 14 A.

Solution used: 0.067 M phosphate (pH 7.4) and
0.015 M Na₂CO₃ mixed in equal parts.

CO₂ tension (as saturated).................................28.2 mm.
Initial CO₂ tension in Clark cell............................28.7 mm.
Temperature of saturation....................................20°C.
No. of saturations...2
"e" of calomel electrode................................0.2493 volts.

CO₂ tension.	Hydrogen electrode.	E. M. F.		Tem-perature.	Barom-eter.	E. M. F. corrected to 1 atmosphere of dry H₂.	pH₂₀°	
		1st filling.	2nd filling.				Electro-metric.	Colori-metric.
mm. Hg		*volts*	*volts*	*°C.*	*mm. Hg*	*volts*	*pH*	*pH*
28.7	A	0.6716	0.6712	20	761	0.6720	7.28	7.28
28.7	B	0.6712	0.6707	20	761	0.6715	7.27	7.27

TABLE II.

Experiment 16 A.

Solution used: 0.067 M phosphate solution (pH 7.35) and
0.015 M Na₂CO₃ mixed in equal parts.

CO₂ tension (as saturated)...............................28.6 mm.
Initial CO₂ tension in Clark cell...........................28.7 mm.
Temperature of saturation....................................20°C.
No. of saturations...2
"e" of calomel electrode................................0.2493 volts.

CO₂ tension.	Hydrogen electrode.	E. M. F.		Tem-perature.	Barom-eter.	E M. F. corrected to 1 atmosphere of dry H₂.	pH₂₀°	
		1st filling	2nd filling.				Electro-metric.	Colori-metric.
mm. Hg		*volts*	*volts*	*°C.*	*mm. Hg*	*volts*	*pH*	*pH*
28.7	A	0.6710	0.6707	20	761	0.6718	7.27	7.27
28.7	B	0.6709	0.6710	20	761	0.6718	7.27	7.27

perature room.   The electrometric determinations were carried out with the same technique and precautions described in the preceding paper.[1]   The system was standardized for each experiment with the

TABLE III.

Experiment 18 A.[*]

Solution used: 0.10 M Na_2HPO_4 ⎫
0.03 M Na_2CO_3 ⎬ mixed in equal parts.

CO$_2$ tension (as saturated)   a   b   c

at 38°......................80.8 mm. 46.7 mm. 27.7 mm.

Initial CO$_2$ tension in Clark cell at 20°..49.2 mm. 29.4 mm. 17.5 mm.

Temperature of saturation..................................38°C.

No. of saturations..2

"e" of calomel electrode at 20°........................0.2493 volts.

| CO$_2$ tension. | H$_2$ electrode. | E. M. F. | | Temperature. | Barometer. | E. M. F. corrected to 1 atmosphere of dry H$_2$. | pH$_{38}$° | |
		1st filling.	2nd filling.				Electrometric.	Colorimetric.
mm Hg		*volts*	*volts*	*°C.*	*mm Hg*	*volts*	*pH*	*pH*
49.2	B	0.6734	0.6747	20	756	0.6759	7.34	7.35
29.4	A	0.6854	0 6870	20	756	0.6879	7.55	7.56
29.4	B	0 6873	0.6874	20	756	0.6883	7 56	7.56
17.5	A	0 6988	0 6983	20	756	0 6990	7.74	7.74
17.5	B	0.6974	0.6984	20	756	0.6991	7.74	7.74

[*] In this experiment the CO$_2$ tensions used in the hydrogen-carbon dioxide mixture for the 20° electrometric determination are calculated to give the same H_2CO_3 concentration *in the solution* at 20° as existed in the solution as equilibrated at 38°.

TABLE IV.

Experiment 24 A.

Solution used: 0.03 M Na_2CO_3 converted to 0.06M $NaHCO_3$ with CO$_2$.

CO$_2$ tension (as saturated)................................40 mm.

Initial CO$_2$ tension in Clark cell...........................40 mm.

Temperature of saturation.............................. ...20°C.

No. of saturations...3

"e" of calomel electrode............................0.2486 volts.

| CO$_2$ tension. | Hydrogen electrode. | E. M. F. | | Temperature. | Barometer. | E. M. F. corrected to 1 atmosphere of dry H$_2$. | pH$_{38}$° | |
		1st filling.	2nd filling.				Electrometric.	Colorimetric.
mm Hg		*volts*	*volts*	*°C.*	*mm Hg*	*volts*	*pH*	*pH*
40	A	0.6962	0.6960	20	753	0.6971	7.72	7.70
40	B	0.6951	0.6957	20	753	0.6968	7.71	7:70

same phosphate solution of pH 7.4 used as the colorimetric standard. The "e" $\left(\text{pH}= \dfrac{\text{E. M. F.} - \text{"e"}}{0.0001984\,T}\right)$ thus obtained was used in calculating the pH of the CO_2-containing solution.[1] This procedure establishes on phosphate solutions, free from CO_2, an identity of pH value for the two methods. The pH values reported are based on the second (refilled) reading, corrected for barometric pressure, CO_2 tension, and vapor pressure to 1 atmosphere of hydrogen.

The results of four experiments are shown in Tables I to IV.

It is evident from these results that, when both colorimetric and gasometric measurements of hydrogen ion concentration are carried out by the technique described, there is complete agreement between the two methods in both carbonate and phosphate-carbonate solutions.

It has recently been reported by Evans[2] that a discrepancy exists between colorimetric and electrometric determinations of the hydrogen ion concentration of fluids containing carbon dioxide. He concluded that the values determined by the colorimetric method were correct and that the electrometric determinations were consistently 0.2 pH too acid, and suggested the use of the correction + 0.2 pH in electrometric determinations. In view of the fact that, with the technique described, we obtain satisfactory agreement between the two methods we feel that the electrometric method must continue to be regarded as the standard.

SUMMARY

The colorimetric and electrometric methods for determining the hydrogen ion concentration of solutions containing carbon dioxide agree when carried out with rigorous precautions to prevent loss of CO_2.

[2] Evans, C. L., *J. Physiol.*, 1921. liv. 353.

A MODIFICATION OF THE CLARK HYDROGEN ELECTRODE VESSEL TO PERMIT ACCURATE TEMPERATURE CONTROL.

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The hydrogen electrode vessel described by Clark[1] is generally accepted as the most convenient type for use with protein solutions and with biological fluids. It is especially suited for use with solutions containing CO_2, and in the 2 cc. form used in this laboratory has proved itself entirely satisfactory for use with blood plasma. Clark recommends that the cell be set up in a constant temperature air bath.

We have observed that in running determinations at 38°, 10 to 15 minutes elapse before solutions introduced at room temperature reach the temperature of the bath. When, as rather easily happens, the outside of the cell is wet, it cannot, of course, attain air temperature until the film of water has evaporated, and the time required becomes longer. Cooling by evaporation would disturb temperature equilibrium even if all materials were at thermostat temperature at the start. There is then a definite possibility of error from trusting to judgment in estimating the time required to attain temperature equilibrium.

Further, we have observed that it has become a rather general practice in many laboratories to use the Clark cell in the open room and to read the temperature from a thermometer suspended either in the KCl reservoir or in the air beside the cell. That this reading often does not represent the actual temperature of the cell contents is evident from Tables I and II, which represent the temperature reading made during average experiments in a room where temperature control was not possible. Errors of 0.01 to 0.03 pH would result from

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[1] Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475; The determination of hydrogen ions, Baltimore, 1920, 128.

TABLE I.*

Temperature Record of pH Measurements at Room Temperature.

Determination.	Time.	Room. Thermometer 1.	KCl cup. Thermometer 2.	Cell A. Thermometer A.	Cell B. Thermometer B.
	a.m.				
1	9.57	22.9	22.5	23.0	23.2
2	10.07	23.0	22.7	23.0	23.0
3	10.15	22.8	22.8	22.9	23.0

Opened window.

4	10.24	21.5	22.7	22.0	22.2
5	10.28	21.0	22.3	21.8	21.8
6	10.36	20.5	21.8	21.1	21.1
7	10.50	20.0	21.0	20.5	20.6
	p.m.				
	12.40				

Solution at 20° added.

8	12.45	22.2		20.4	20.5
9	12.55	22.0		21.0	21.0

TABLE II.*

Temperature Record of pH Measurements in Thermostat Set for 37°C.

Determination.	Time.	Thermometer 3.	Thermometer 4.	Cell C. Thermometer C.	Cell D. Thermometer D.
	p.m.				
10	2.46	37.2	37.5	36.0	35.0
11	2.52	37.2	37.5	37.0	37.1

Opened door 1 minute to make contact.

12	3.54	36.2	37.5	37.0	37.0
13	3.60	37.2	37.5	37.0	37.0

	4.00	Put in solution at 22°C.			
14	4.08	37.0	37.5	36.0	36.0
15	4.13	37.2	37.5	37.0	37.0

* Readings were made with Thermometers 1 and 3 hanging between the two electrode vessels about 1½ inches from each and at the same level. Thermometer 2 was inserted in the KCl cup along side of the connection of the calomel cell and Thermometer 4 in the thermostat was at the back over the lamps. Thermometers A, B, C, and D were inserted in the electrode vessels in the manner described in the text.

the use of the temperature as read from the thermometer suspended between the cells.

The writer has found that errors from these sources may be advantageously obviated by inserting a thermometer into the solution. For this purpose an extra opening in the Clark cell is prepared and a
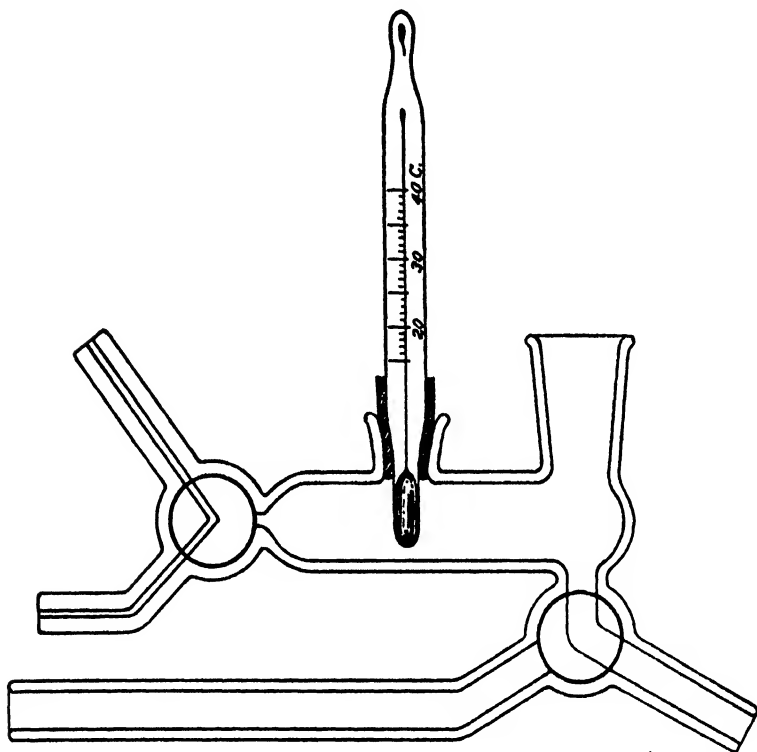
FIG. 1.

short calibrated thermometer introduced as shown in Fig. 1. The thermometer is carried in pure rubber tubing but could, of course, be ground in.

The construction is evident from the figure, but with the 2 cc. cells which we use care must be taken to make the thermometer bulb so small that it does not obstruct the flow of solution during the rocking of the electrode. The bulb from the shoulder to the tip must be shorter than the inside diameter of the tube, else it is difficult to insert the thermometer without dead space.

For the small cells we use, we specify that the opening to receive the thermometer be exactly round, 8.5 mm. diameter at the top, and 7.5 mm. at the bottom. The opening is about 5 mm. high The thermometer must be 5 mm. in diameter and about 48 mm. long over all with mercury bulb 8 mm. long tapering straight from the shoulder to 1 to 2 mm. at the tip. There must be no constriction or bulge at the shoulder.[2]

It is felt that this modification of the Clark cell facilitates its use even in a carefully controlled thermostat and markedly increases accuracy when it is used in the ordinary room.

[2] The cells and thermometer were made for us by Wm. Weigand, 141 Lexington Ave., New York.

ON THE MEASUREMENT OF BUFFER VALUES AND ON THE RELATIONSHIP OF BUFFER VALUE TO THE DISSOCIATION CONSTANT OF THE BUFFER AND THE CONCENTRATION AND REACTION OF THE BUFFER SOLUTION.*

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CONTENTS.

The Nature and Mode of Action of Buffers.

In his chapter on buffer action Clark (1920) has traced the development of the knowledge of buffers,[1] which we owe mainly to the work

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* The present paper constitutes the theoretical basis for experimental work, the results of which will follow.

[1] Clark (1920), p. 30.

of L. J. Henderson (1908), and of Sörensen (1912), and has summarized the resultant conception in the statement: "By buffer action we mean the ability of a solution to resist change in pH through the addition or loss of alkali or acid." Reduced to the form of a definition this conception may be expressed as follows: *Buffers are substances which by their presence in solution increase the amount of acid or alkali that must be added to cause unit change in pH.* As will appear later, change in the logarithmic pH unit affords a more convenient measure of buffer effect than change in [H$^+$].

The most efficient buffers, at reactions within the usual range of biological significance, are mixtures of weak acids or weak bases and their salts. Their buffer effect is due to the relatively slight extent to which they undergo electrolytic dissociation, as compared with the almost completely dissociated strong acids and bases.

If we add a strong acid, HCl for example, to a mixture of the weak buffer acid, Ha, and its alkali salt, Ba, reaction occurs with approximate completeness from left to right according to the equation

$$HCl + Ba = Ha + BCl$$

(To represent the weak buffer acid the formula Ha is used to distinguish it from HA, which will be used to indicate a strong, approximately entirely dissociated acid.) In this reaction the strong acid combines with an equivalent of buffer salt and sets free an equivalent of weak buffer acid, Ha. The latter, instead of dissociating like HCl almost entirely into hydrions and anions, dissociates only slightly. Hence the increase in hydrion concentration, and the change in pH, is only a fraction of that which would have been caused by adding the same amount of HCl to an unbuffered solution.

The amount of change that does occur depends on the extent to which the buffer acid set free dissociates according to the equation Ha = H$^+$ + a'. If 1 per cent of Ha dissociates into H$^+$ and a', the H$^+$ increase will be approximately 0.01 of that which would have been caused by adding the HCl to an unbuffered solution.

Similarly, if we add a strong base, BOH, such as KOH or NaOH, to the buffer solution, an equivalent of buffer acid in the Ba, Ha mixture is neutralized according to the equation

$$BOH + Ha = Ba + H_2O$$
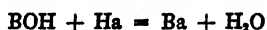
The decrease in H^+ concentration due to neutralization of a given amount of Ha is again dependent on the extent to which the Ha had been dissociated, since only the H^+ ions owing their presence to that dissociation have disappeared.

It is evident from the above that the Ba of the buffer mixture reacts when acid is added, and the Ha when alkali is added, and therefore that both Ba and Ha are necessary if the buffer mixture is to offer resistance to reaction change by addition of either acid or alkali. As will be seen later, if ability to minimize the proportion by which any given H^+ concentration is changed is taken as a measure of buffer action, a buffer mixture has its maximum efficiency when Ha = Ba, half the buffer being free, half in the form of its salt.

When the buffer mixture is composed of a weak base, bOH, and its salt, bA, it can be shown similarly that the amount of change in H^+ or OH' concentration caused by addition of acid or alkali depends on the extent to which bOH dissociates into b^+ and OH' ions.

Since weak acids and bases obey in their dissociation the simple law of mass action, and since their buffer action is dependent on the extent of their dissociation, it follows that the quantitative relationships governing buffer action are capable of formulation from the mass law.

By means of such formulation, L. J. Henderson (1908) has shown that buffer acids most efficient in maintaining a neutral reaction of $[H^+] = [OH'] = 10^{-7}$ are such as have dissociation constants most nearly equal to 10^{-7}. Clark (1920) has pointed out[2] "that it is only within a certain zone of $\log \dfrac{1}{[H^+]}$ that a mixture of an acid with its salt produces a stabilized hydrogen ion concentration or pH." The writer has recently indicated (1921, a) a mode for the mathematical proof that in general, if buffer efficiency be considered as ability to minimize change in pH, that is, proportional change in $[H^+]$, any buffer salt of a weak acid is most efficient when [Ha] and [Ba] are equal, under which conditions pH = pK_a'.[3]

[2] Clark (1920), p. 19.

[3] pK_a' is an expression introduced by Hasselbalch (1917) to indicate the negative logarithm of K_a', the value of K_a' being $\dfrac{K_a}{\gamma}$ where K_a is the dissociation constant of

Unit for Measurement of Buffer Values.

Thus far, however, there has appeared in the literature no satisfactory mode of expressing, over the zone of pH at which buffers act, the quantitative relationships of buffer effect to the dissociation constant of the buffer and the reaction: in fact there has been no unit for the numerical expression of buffer effect. It is the purpose of the present paper by means of such a unit to attain quantitative measurement and expression of buffer effects, and to derive from the mass law the above mentioned relationships.

The unit adopted is the differential ratio $\dfrac{dB}{dpH}$, expressing the relationship between the increment (in gram equivalents per liter) of strong base B added to a buffer solution and the resultant increment in pH. Increment of strong acid is equivalent to a negative increment of base, or $-dB$. In these terms *a solution has a buffer value of 1 when a liter will take up 1 gram equivalent of strong acid or alkali per unit change in pH.*

If base is added to a solution, pH is increased, so that both dB and dpH are positive. If acid is added both dB and dpH are negative. The ratio $\dfrac{dB}{dpH}$ is, therefore, always a positive numerical value. If one solution has twice the buffer value of a second solution, twice as much base or acid is required to change the pH by a given small amount, for example 0.1 and therefore the value $\dfrac{dB}{dpH}$ is twice as great in the first solution as in the second. For convenience we shall use at times the letter β to indicate the ratio $\dfrac{dB}{dpH}$.

The significance of the ratio $\dfrac{dB}{dpH}$ as a measure of buffer effect is illustrated by Fig. 1. In place of dB and dpH, infinitesimal incre-

the buffer acid [Ha], γ the degree of dissociation of its salt [Ba] into [B$^+$] and [a$'$] ions. Hasselbalch actually used the form K_1. We have altered it to K' in order to facilitate differentiating between K_a and K'_b, derived from the acid and basic dissociation constants customarily designated as K_a and K_b respectively.

ments, we there use measurable increments ΔB and ΔpH, which, if not too great, serve our purpose nearly as well. In order to increase the pH of Solution 1 from 3 to 4, 0.1 gram molecule of NaOH per
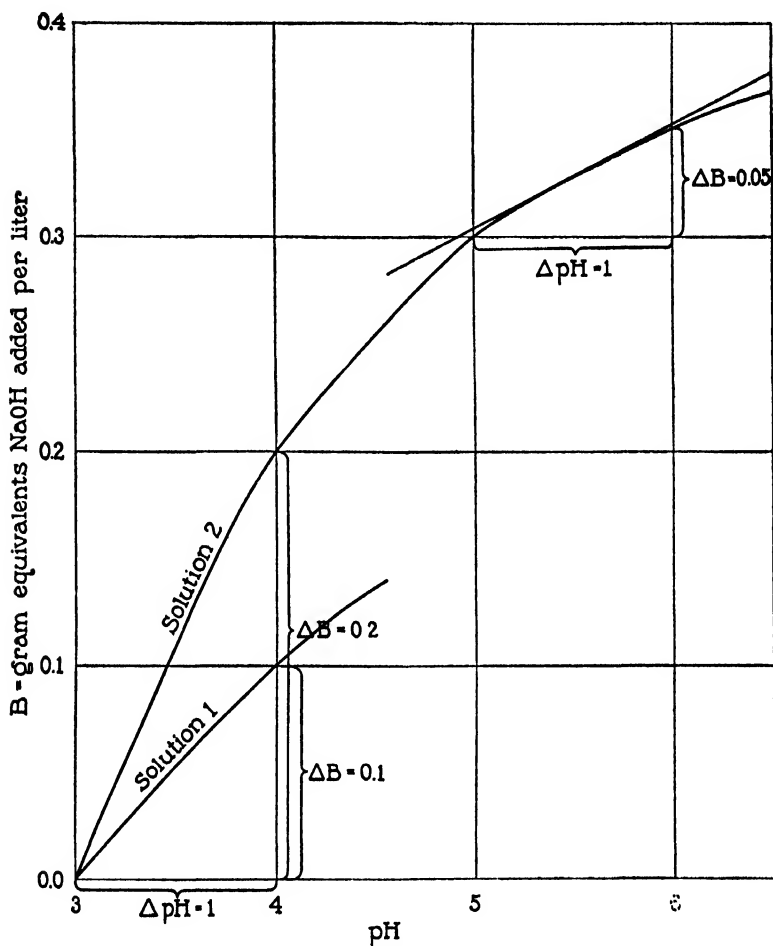
FIG. 1.

liter is required; therefore $\Delta B = 0.1$. Since $\Delta pH = 1$ (the increase in pH caused by the change from pH 3 to 4), the approximate buffer value of Solution 1 at the mean reaction of pH 3.5 is $\beta = \dfrac{\Delta B}{\Delta pH} = \dfrac{0.1}{1.0}$

$= 0.1$.

In order to increase the pH of Solution *2* from pH *3* to pH *4, 0.2 gram molecule of NaOH per liter is required; therefore, for ∆pH = 1, ∆B = 0.2; and, at mean pH 3.5, $\beta = \dfrac{0.2}{1.0} = 0.2$.

Let us now follow the curve of Solution 2 further along until it covers the range of pH 5 to 6. Because the curve has become less steep, we find that the ∆B accompanying the pH increase from 5 to 6 is only 0.05. Consequently, Solution 2, which at pH 3.5 had a buffer value of $\dfrac{\Delta B}{\Delta pH} = 0.2$, has at pH 5.5 only one of $\dfrac{\Delta B}{\Delta pH} = 0.05$.

In place of·measuring the ∆B and ∆pH values for equal distances on both sides of the pH at which we wish to determine the value of $\dfrac{dB}{dpH}$ we may draw a tangent to the curve at the indicated pH and measure the slope of the tangent. Thus, in the case of Solution 2, we may draw a tangent to the curve at pH 5.5. Since the tangent, being a straight line, has the same slope at all points, we may prolong it as much as we wish in order to determine its slope conveniently. For example, we make the measurements between pH 5 and 6.5. At these points the tangent cuts the B lines at 0.303 and 0.376 respectively, the difference being 0.073. The slope of the tangent, or the value of $\dfrac{dB}{dpH}$ at pH 5.5, is therefore $\dfrac{0.073}{1.500} = 0.0486$, which approximates the value $\dfrac{\Delta B}{\Delta pH} = 0.05$ obtained above. The tangent, if accurately drawn, gives the exact $\dfrac{dB}{dpH}$ value at its point of contact with the curve, and therefore has some theoretical advantage over the use of the measurable increments ∆B and ∆pH, which indicate the approximate buffer value at the mean pH over the portion of curve measured.

The curve of Solution 2 illustrates the fact that a given solution may have a different buffer value at different reactions, and that one may speak in exact terms only of its buffer value at a definite pH, or of its average buffer value over a certain pH range. The manner in which the buffer value of a solution containing a single buffer acid and its salt changes with varying pH is shown by Figs. 4 and 9.

Since a solution may have a different buffer value at every pH, rigid accuracy would require that it be measured by the ratio $\dfrac{dB}{dpH}$, of the infinitesimal increments dB and dpH. Actually, as may be seen from Fig. 4, increments ΔB and ΔpH of sufficient size to be measured may be usually employed with fair accuracy.

The Buffer Value of Water Plus Only Strong Acid or Alkali.

When strong acid or alkali is added to water the solution displays a certain buffer value. If it did not, each addition of acid or alkali would cause infinite change in pH.

Let us assume that a strong base completely dissociating into the ions [B+] and [OH'] is added to water. Then the increment dB, in base, is equal to the increment d[OH'], in [OH']. Consequently for $\dfrac{dB}{dpH}$ we may write $\dfrac{d\,[OH']}{dpH}$.

$$pH = -\log [H^+] = -\log \frac{k_w}{[OH']} = \log [OH'] - \log k_w, \text{ when } k_w$$

is the water constant [OH'] $\times$ [H+]. Hence dpH = d log [OH']. Therefore

$$(1) \qquad \frac{dB}{dpH} = \frac{d\,[OH']}{d \log [OH']} = \frac{[OH']}{0.4343} = 2.3\,[OH']$$

Similarly, if we add to water a strong acid, completely dissociated into [H+] ions and anions, such addition constitutes a negative addition of base. The increment in acid is equal to that in [H+], that is $-dB = d[H^+]$.

$$(2) \qquad \frac{dB}{dpH} = \frac{-\,d\,[H^+]}{-\,d \log [H^+]} = \frac{[H^+]}{0.4343} = 2.3\,[H^+]$$

If we add the two effects, expressed by Equations 1 and 2, respectively, we express the total buffer value of water plus completely dissociated acid or alkali at all pH's, as shown in Equation 3.

$$(3) \qquad \frac{dB}{dpH} = 2.3\,([H^+] + [OH'])$$

At any given [H+] or [OH'], for each gram equivalent of hydrion or hydroxyl ion present, completely dissociating base or acid must

be added at the rate of 2.3 gram equivalents per liter per unit change in pH effected.

The relationships expressed in Equation 3 are shown in Figs. 2 and 3. It is obvious from them that over the reaction range usually significant in animal or plant physiology, pH 3 to 11, the buffer value of completely dissociated acid or alkali is slight, too slight in fact to show graphically on charts of the scale used, but that at both ends of this range it becomes significant.

The actual dissociation of a strong acid, such as HCl, or a strong base, such as NaOH, is only approximately complete at finite dilutions. If we express the fraction of BOH dissociated into $[B^+]$ and $[OH']$ by γ_B, $[OH'] = \gamma_B [BOH] = \gamma_B B$, $B = \dfrac{[OH']}{\gamma_B}$, and $dB = \dfrac{d[OH']}{\gamma_B}$. Similarly $dB = \dfrac{-d[H^+]}{\gamma_A}$ if γ_A represents the degree of dissociation of the strong acid. Consequently, if instead of ideal, completely dissociated strong base and acid, our additions to water are of actual, not quite completely dissociated strong base and acid, γ_B and γ_A being a little less than 1, instead of Equation 3, we have the slightly different Equation 4.

$$(4) \qquad \frac{dB}{dpH} = 2.3 \left(\frac{[H^+]}{\gamma_A} + \frac{[OH']}{\gamma_B} \right)$$

The fact that this equation is accurate is illustrated by the following example. 0.1 N HCl is, according to the conductivity data of Noyes and Falk, 92.3 per cent dissociated at 20°. $[H^+] = 0.0923$ N; pH $= 1.035$. If we increase the HCl to 0.11 N, $[H^+]$ increases to $0.0923 \times 1.10 = 0.1015$ (the dissociation does not change significantly), and pH falls to 0.994. From these measured changes, ΔB and Δ pH, we calculate the approximate β value at the mean pH between 1.035 and 0.994.

$$\beta = \frac{\Delta B}{\Delta pH} = \frac{0.10 - 0.11}{0.994 - 1.035} = \frac{-0.01}{-0.041} = 0.243$$

The mean pH of the range, pH 1.035 to 0.994, is 1.015. The corresponding $[H^+]$ is 0.0966 ($[OH']$ is negligible). The buffer value of the solution, at pH 0.994 calculated by Equation 4, with $\gamma_A = 0.923$, is

$$\beta = \frac{dB}{dpH} = 2.3 \frac{0.0966}{0.923} = 0.241$$

Neglecting γ_A and calculating by Equation 3 we would obtain

$$\beta = 2.3 \times 0.0966 = 0.222$$
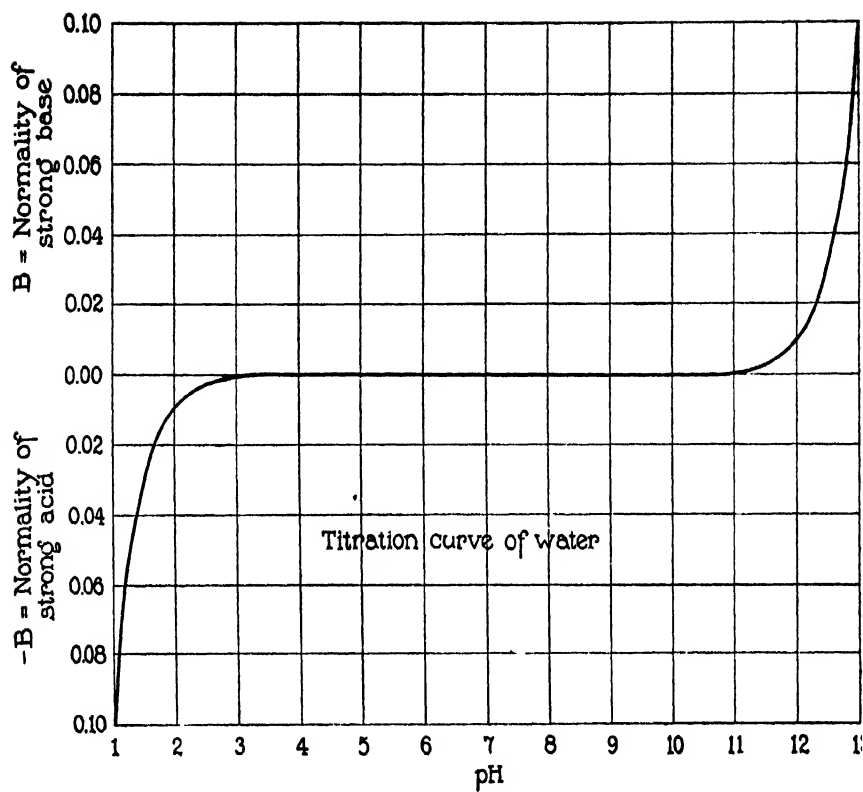
Fig. 2.
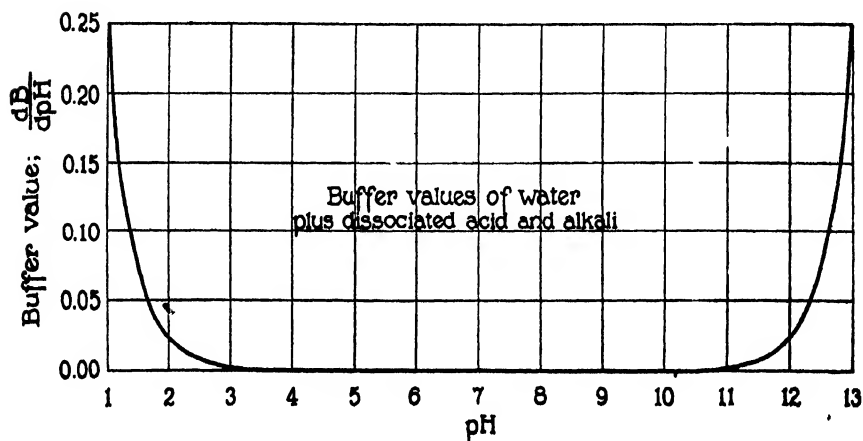
Fig. 3.

For HCl solutions more dilute than 0.1 N, γ_A approaches more nearly 1, and the difference between results calculated by Equations 3 and 4, respectively, becomes still less than in this example.

The buffer action of a strong, completely dissociated acid or base is different in its mechanism from the buffer action of a weak, slightly dissociated acid or base, such as was discussed in the first section of this paper, and is about to be considered quantitatively in the next section. The buffer action of the weak acid or base involves repression of part of the $[H^+]$ change that would be caused by addition of acid or alkali to an unbuffered solution. With repression of $[H^+]$ change there is also, of course, an accompanying repression of the *proportion* by which $[H^+]$ is altered, and it is this proportional $[H^+]$ change that is a linear function of the log $[H^+]$ or pH change which we have adopted to measure buffer effect.

The fact that changes in log $[H^+]$, and in pH, are linear measures of the proportion by which $[H^+]$ is changed may be demonstrated as follows. If d $[H^+]$ is the increment in $[H^+]$, the proportion of itself by which $[H^+]$ is increased is $\dfrac{d\,[H^+]}{[H^+]}$. If an increment, $-dB$, of strong acid is added, with resultant absolute change in $[H^+]$ of $d[H^+]$, we have $\dfrac{-\,dB}{\dfrac{d\,[H^+]}{[H^+]}}$ as the ratio between added acid and the proportion by which $[H^+]$ is increased. But in general,

$$\frac{dx}{\dfrac{dy}{y}} = \frac{dx}{d\log_e y} = \log_{10} e\,\frac{dx}{\log_{10} y}$$

$$\text{Hence } \frac{-\,dB}{\dfrac{d\,[H^+]}{[H^+]}} = -\log_{10} e\,\frac{dB}{\log_{10}[H^+]} = 0.4343\,\frac{dB}{dpH}$$

$$dpH = -\,0.4343\,\frac{d\,[H^+]}{[H^+]}$$

It is obvious from the right-hand member of the last equation that as factors of dpH we must consider not only $d[H^+]$, but also $[H^+]$.

The presence of a completely dissociated acid or base, would repress *only* the proportion by which a given further addition would alter the $[H^+]$. The absolute $[H^+]$ change would be practically unaffected by strong acid already present before this addition: only

the $[H^+]$ factor of the $\dfrac{d[H^+]}{[H^+]}$ ratio would be affected.   The greater the amount of $[H^+]$ or $[OH']$ (or completely dissociated acid or alkali) present, however, the greater the further amount required to be added or removed to change the original to 10-fold or $\frac{1}{10}$ its own value, and thereby cause unit change in pH.   And this further amount is our measure of buffer value.   So, if we adopt pH change as our unit of reaction shift, we must assign a definite buffer effect to the mere mass of $[H^+]$ or $[OH']$ ions present, which, by its inertia, so to speak, represses pH change.   This effect we may term *the buffer effect of the dissociated base or acid, or of the $[H^+]$ or $[OH']$ concentration.*

In a sense, therefore, the adoption of $\dfrac{dB}{dpH}$ as the unit of buffer value has broadened the conception of buffer action outlined in the introduction.   Buffer actions have there been discussed as processes repressing $[H^+]$ changes by replacement of the added, more or less strongly dissociated acids or bases with the weak, undissociated acids or bases of the buffers.   To this action we must add the "inertia" buffer effect peculiar to dissociated acids and bases, which repress the proportional, though not the absolute, $[H^+]$ change, and which attain importance when $[H^+]$ or $[OH']$ exceeds about 10^{-3} (when pH is less than 3 or greater than 11).   As will appear later (Equations 17 and 35) the total buffer value of a solution is represented by the sum of the buffer effects due, respectively, to dissociated and to undissociated acids or bases present.   The two effects may both be important in a buffer forming a very acid solution, such as H_3PO_4, or forming a highly alkaline one, such as Na_3PO_4.

The Buffer Value of a Solution of a Weak Monovalent Acid and Its Salt.

Fundamental Mass Law Equations.—The fundamental equation derived from the mass action law of Guldberg and Waage as applied by Arrhenius to the dissociation of electrolytes, and shown by Ostwald (1888) to hold for dilute solutions of weak acids is

(5) $$K_a = \frac{[H^+] \times [a']}{[Ha]}$$

where [Ha] is the undissociated portion of the acid, [H⁺] the hydrion concentration, [a′] the concentration of anions formed by dissociation of the acid, and K_a the dissociation constant of the acid.

This equation has been applied in two forms. It was used by Ostwald (1888) for pure solutions of weak acids in the form

$$(6) \qquad\qquad K_a = \frac{[H^+]^2}{[Ha]}$$

The theoretical accuracy of Equation 6 for such solutions follows from the fact that both hydrions and anions must arise in equal numbers from the dissociation of [Ha], when the latter is their sole significant source.

For solutions containing not only the free acid, but also its salt, [Ba], Equation 5 was converted by L. J. Henderson (1908, 1909) into the form

$$(7) \qquad\qquad K_a = \frac{[H^+]\,\gamma_s\,[Ba]}{[Ha]}$$

[Ha] = concentration of free undissociated acid.
[Ba] = total concentration of salt, dissociated or undissociated.
γ_s = fraction of [Ba] dissociated into [B⁺] and [a′].
γ_s[Ba] = concentration of a′ formed by dissociation of [Ba].

The salt is usually 80 per cent or more dissociated (γ_s = 0.8 to 1.0) while the free acid is dissociated to a relatively negligible extent into [H⁺] and [a′]. The slight amount of [a′], equal to the [H⁺], arising from dissociation of the free acid is, at reactions covering a considerable zone on both sides of neutrality, negligible in comparison to the amount formed from the salt. Consequently, over a pH range of about 7±3 or 4, γ_s [Ba] can be inserted, with a negligible error in place of [a′] in Equation 5. (A certain inconsistency in symbols occurs through the use of [Ha] to represent only the undissociated part of the acid, while [Ba] represents the total salt, dissociated and undissociated. It has seemed preferable, however, to retain [Ba] with this significance, partly because it has been used in this manner in the literature since Henderson introduced his equation, partly because it has the advantage of being a self-explanatory symbol.)

Hasselbalch (1917) expressed Henderson's equation in the logarithmic form

(8)
$$pH = pK_a' + \log \frac{[Ba]}{[Ha]}$$

pK_a' being the negative logarithm of K_a', while $K_a' = \frac{K_a}{\gamma_s}$.

Equation 7, and its equivalent, Equation 8, hold for all $[H^+]$ values that are found in body fluids and excretions (except perhaps gastric juice) since the conditions are fulfilled that $[H^+]$ and $[OH']$ are both small (in the neighborhood of 10^{-7} in the internal fluids, and not exceeding 10^{-5} in urine), and the concentration of anions formed by dissociation of the buffer salts present is sufficient to make $\gamma_s [Ba]$ the only factor that requires consideration in estimating $[a']$. Equations 7 and 8 are therefore accurate for the physiological range for which Henderson used them, and may in fact be used over a still wider reaction range. The limitations of this range will be considered later.

Differentiation of Henderson's Equation in Order to Calculate the Buffer Values of Weak Acids.—In order to obtain the buffer value $\frac{dB}{dpH}$, in terms of the buffer concentration and reaction, we convert Equation 7 into the form

(9)
$$K_a = \frac{[H^+] \gamma_s B}{C - B}$$

    C = total molecular concentration of buffer acid before addition of base.

    B = gram equivalents of strong base added.

C − B = [HA]

Within the range of validity of Henderson's equation, all the BOH added takes the form of the buffer salt, [Ba], the base remaining free as BOH being negligible. Therefore, we may substitute B for [Ba] in Equation 7. Furthermore, since all save a negligible portion of the buffer acid is in the form either of free undissociated acid, [Ha], (the negligible residue is dissociated into $[H^+]$ and $[a']$), or salt, [Ba], we may substitute C − B for [Ha]. With these two substitutions Equation 7 is changed into Equation 9.

Solving Equation 9 for B we have

(10)
$$B = \frac{K_a C}{K_a + \gamma_s [H^+]} = \frac{K_a' C}{K_a' + [H^+]} \text{ (where } K_a' \gamma_s = K_a)$$

$$\frac{dB}{dpH} = -\frac{dB}{d \log [H^+]} = -\frac{[H^+]}{0.4343} \times \frac{dB}{d [H^+]} = -2.3 [H^+] \frac{dB}{d [H^+]}$$

Differentiating the first form of Equation 10, $B = \dfrac{K_a\,C}{K_a + \gamma_s\,[H^+]}$, we have

$$\frac{dB}{d\,[H^+]} = -\,\frac{K_a\,\gamma_s\,C}{(K_a + \gamma_s\,[H^+])^2}$$

From which we obtain, by multiplying $\dfrac{dB}{d\,[H^+]}$ by $-2.3\,[H^+]$,

(11) $\qquad\qquad \dfrac{dB}{dpH} = \dfrac{2.3\,K_a\,\gamma_s\,C\,[H^+]}{(K_a + \gamma_s\,[H^+])^2}$

(12) $\qquad\qquad\quad = \dfrac{2.3\,K_a'\,C\,[H^+]}{(K_a' + [H^+])^2}$

(13) $\qquad\qquad\quad = \dfrac{2.3\,[Ba]\,[H^+]}{(K_a' + [H^+])}$

(14) $\qquad\qquad\quad = \dfrac{2.3\,[Ba]\,[Ha]}{C}$

Equivalent expressions for the buffer value $\dfrac{dB}{dpH}$, or β, for weak acids.

Equation 12 is derived from Equation 11 by substituting $K_a'\,\gamma_s$ for its equivalent K_a, or by differentiation of the second form of Equation 10, in which this substitution is already made. Equation 13 is derived from Equation 12 by substituting $[Ba]$ for $\dfrac{K_a'\,C}{K_a' + [H^+]}$. $\Big($Henderson's equation may be written $K_a'\,(C - [Ba]) = [H^+]\,[Ba]$, whence $[Ba] = \dfrac{K_a'\,C}{K_a' + [H^+]}\Big)$. Equation 14 is derived from Equation 13 by substituting $[Ha]$ for $\dfrac{[H^+]\,C}{K_a' + [H^+]}$. (If Henderson's equation is written $K_a'\,[Ha] = [H^+]\,(C - [Ha])$ it yields this value for $[Ha]$).

We have tested the approximate accuracy of Equation 14 (and therefore of its equavalents, Equations 11, 12, and 13) against experimental results by applying it to Clark and Lubs' data for KH_2PO_4 — $KHPO_4$ mixtures (1916), the pH values being electrometrically determined by Clark for varying $[Ba] : [Ha]$, or K $(KHPO_4)$: H $(KHPO_4)$, ratios. Considering the short intervals used ($\Delta pH = 0.2$), the agreement between the theoretically calculated $\dfrac{dB}{dpH}$ values and

those approximated from the experimental ΔB and ΔpH figures is as satisfactory as could be expected from this mode of calculation.

The data of Table I are expressed graphically in Fig. 4, the curve being calculated from Equation 8 with $pK_a' = 6.85$. Clark's experimentally determined points are indicated by crosses.

It is evident from Equation 12 that the buffer effect expressed in terms of K_a and $[H^+]$ is proportional to the total molecular concentration $[C]$ of the buffer. We may therefore divide the derivative by $[C]$, in order to determine the *molecular buffer value* of a solution, or the buffer value which an M solution of it would have. This unit may be used for comparison of different buffers. It becomes

$$(15) \qquad \frac{dB}{CdpH} = \frac{2.3\,K_a'\,[H^+]}{([H^+] + K_a')^2} = \beta_M$$

We shall hereafter refer for convenience to the absolute buffer value as β, the molecular buffer value as β_M. The relationship between the two is

$$(16) \qquad \frac{\beta}{C} = \beta_M$$

The total buffer value of a solution of a weak acid to which both strong acid and alkali are added in amounts not limited to equivalence with the buffer acid, may be expressed as the β of the buffer plus the β of the free dissociated acid or alkali. This is expressed by combining Equations 3 and 12 in Equation 17.

$$(17) \qquad \beta = 2.3 \left(\frac{K_a'\,C\,[H^+]}{(K_a' + [H^+])^2} + [H^+] + [OH'] \right)$$

For acetic acid ($pK_a' = 4.6$) in 0.2 and 0.1 M concentrations, the total buffer value of the solution from pH 1 to 13 is indicated by Fig. 5. Where the buffer effects of the acetate and hydrion overlap, the separate β values are indicated by broken lines, the actual total β values of the solution by continuous lines.

The use of $\frac{\beta}{C}$ as β_M is obviously permissible only where $[H^+]$ and $[OH']$ are negligible in comparison with $\dfrac{K_a'\,C\,[H^+]}{(K_a' + [H^+])^2}$ which is the case, with $C = 0.1$ M, between pH values of about 3 and 11.

TABLE I.

Values of $\dfrac{\Delta B}{\Delta pH}$, as Estimated from Values of ΔB and ΔpH Taken Directly from W. M. Clark's Curves for 0.05 M KH_2PO_4 Plus NaOH, Compared with Values of $\dfrac{dB}{dpH}$ Calculated by Equation 14.

Phosphate mixtures from Clark.
C = 0.05 M

pH	Mean pH.	Δ pH	[Ba]	pKₐ′*	Mean [Ba].	Mean [Ha].	Δ B	$\frac{\Delta B}{\Delta pH}$	$\frac{dB}{dpH}$ calculated as 46 [Ha] [Ba] from mean [Ha] and [Ba]†	Molecular buffer value β_M. $\frac{\Delta B}{0\,05\,\Delta pH}$	$\frac{dB}{0.05\,dpH}$
5.8			0.0037	6.90							
	5.9	0.2			0.0047	0.0453	0.0020	0.0100	0.0098	0.200	0.198
6.0			0.0057	6.89							
	6.1	0.2			0.0062	0.0438	0.0029	0.0145	0.0125	0.290	0.250
6.2			0.0086	6.88							
	6.3	0.2			0.0106	0.0394	0.0040	0.0200	0.0192	0.400	0.384
6.4			0.0126	6.87							
	6.5	0.2			0.0152	0.0348	0.0052	0.0260	0.0245	0.520	0.490
6.6			0.0178	6.86							
	6.7	0.2			0.0207	0.0293	0.0058	0.0290	0.0279	0.580	0.558
,6.8			0.0236	6.85							
	6.9	0.2			0.0266	0.0234	0.0060	0.0300	0.0287	0.600	0.574
7.0			0.0296	6.84							
	7.1	0.2			0.0323	0.0177	0.0054	0.0270	0.0263	0.540	0.526
7.2			0.0350	6.83							
	7.3	0.2			0.0372	0.0123	0.0045	0.0225	0.0211	0.450	0.422
7.4			0.0395	6.83							
	7.5	0.2			0.0411	0.0089	0.0033	0.0165	0.0168	0.330	0.336
7.6			0.0428	6.83							
	7.7	0.2			0.0440	0.0060	0.0024	0.0120	0.0121	0.240	0.242
7.8			0.0452	6.83							
	7.9	0.2			0.0450	0.0050	0.0016	0.0080	0.0104	0.160	0.208
8.0			0.0468	6.83							

* Calculated as $pK_a' = pH - \log \dfrac{[Ba]}{0.05 - [Ba]}$.

† $\dfrac{2.3}{C} = \dfrac{2.3}{0.05} = 46$ (Equation 14).
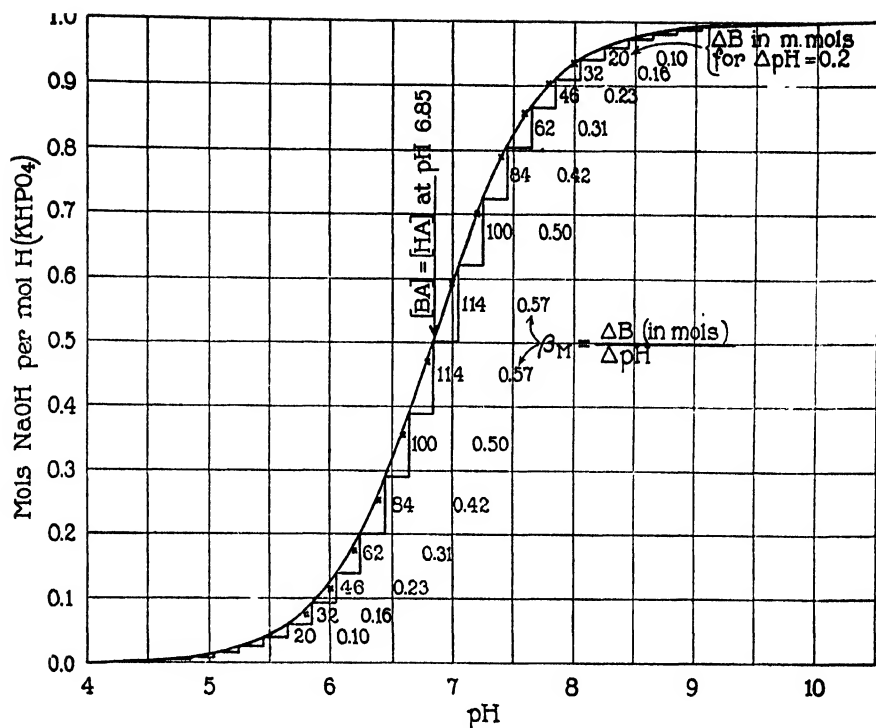
FIG. 4. Titration curve of H(KHPO$_4$) plus NaOH. Curve is calculated from equation, pH $= 6.85 + \log \dfrac{\text{Na(KHPO}_4)}{\text{H(KHPO}_4)}$. Crosses are from experimental data of Clark and Lubs.
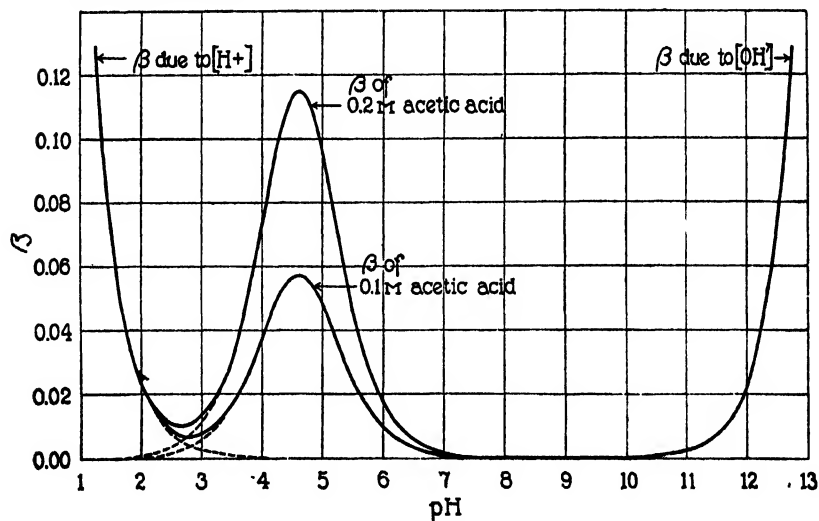
FIG. 5.

Second Derivative of Henderson's Equation. Point of Maximum Buffer Value.—To find the conditions under which a mixture of a weak acid and its salt exerts maximum buffer effect, we repeat the differentiation. From Equation 12, $\beta_M = 2.3\,K_a'\,\dfrac{[H^+]}{(K_a' + [H^+])^2}$

$$\frac{d\beta_M}{d[H^+]} = 2.3\,K_a'\,\frac{[K_a']^2 - [H^+]^2}{(K_a' + [H^+])^4} = 2.3\,K_a'\,\frac{K_a' - [H^+]}{(K_a' + [H^+])^3}$$

(18)
$$\frac{d\beta_M}{dpH} = -2.3[H^+]\frac{d\beta_M}{d[H^+]} = \overline{2.3}^2 K_a'[H^+]\frac{[H^+] - K_a'}{(K_a' + [H^+])^3}$$

From Equation 18 it is evident that β_M, the molecular buffer value, has either a maximum or a minimum value when $[H^+] = K_a'$, since this is the condition for making $\dfrac{d\beta_M}{dpH}$ equal to zero. The fact that maximum, not minimum, value of β_M occurs when $[H^+] = K'$ is obvious from an inspection of the curve of Fig. 4. The occurrence of the maximum at this point was somewhat less rigidly shown by the writer in a former paper (1921, *a*).

At the maximum point, since $[H^+] = K_a'$, we have from Equation 15

(19)
$$\beta_M = \frac{2.3[H^+]^2}{(2H^+)^2} = \frac{2.3}{4} = 0.575$$

For every buffer this slope indicates a value of $[Ha] = [Ba]$, since when $K_a' = [H^+]$, $[Ha]$ must equal $[Ba]$, as is evident when Henderson's equation is put into the form $[Ba][H^+] = [Ha]\,K_a'$.

The relationships developed lead to the following conclusions, an example of each of which may be obtained from Table I and Fig. 4.

1. All monovalent acid buffers acting within the range of validity of Henderson's equation (with pK_a' between about 4 and 10) have the same maximum molecular buffer value *viz.* $\beta_M = 0.575$, which they exert when $[H^+] = K_a'$, or $pH = pK_a'$. (In the case of $H(KHPO_4)$, used in Table I, $pK_a' = 6.85$).

2. At this pH, $[Ba] = [Ha] = \dfrac{C}{2}$, that is, half the buffer acid is free, half in the form of alkali salt.

3. If the point of maximum slope be determined in a buffer curve with increments of added alkali as ordinates and pH values as abscissæ, one may calculate the molecular concentration of the buffer by Equation 20, which is a rearrangement of Equation 19.

$$(20) \qquad C = \frac{\beta}{0.575}$$

i.e., the molecular concentration of the buffer is found by dividing the tangent of the maximum slope of the titration curve by 0.575.

Equation 19 holds even for polybasic acids, provided the dissociation constants of the different acid hydrogen atoms are far enough apart so that, at the pH of its maximum buffer efficiency, each one exerts a buffer effect relatively uninfluenced by the others. If two buffer acids are in equal concentration in the same solution, their pK' values must be at least 2.5 apart in order that the buffer effect of each may be less than 1 per cent that of the other at the latter's maximum. This is the case with phosphoric acid, of which the three pK' values are 2.0, 6.8, and 12.0, as seen from Fig. 6. The manner in which polybasic acids may be handled when their buffer effects overlap is discussed later.

It is to be noted that the relationship [Ba] = [Ha] at a point of maximum buffer value in such a polyvalent acid buffer refers to [Ba] only as the amount of base replacing the hydrogen of the particular acid group which is acting as a buffer at the existing pH.

4. The pH at the point of maximum slope of the buffer curve indicates the value of pK_a'.

5. For β values other than the maximum K_a' may be calculated by solving Equation 12 for K_a'. We obtain thus from Equation 12

$$(22) \qquad K_a' = [H^+] \frac{C - 0.8686\beta_M \pm \sqrt{(0.8686\beta_M - C)^2 - 0.756\beta_M^2}}{0.8686\beta_M}$$

Of the two K_a' values obtained by Equation 22, that is correct which is identical with [H+] at the point of maximum β value, or the β_M value of 0.575. While ordinarily there is no advantage in using values of β or β_M, and Equation 22, rather than values of [Ba] and Equation 7 in calculating an acid dissociation constant, yet, there may be occasions in which β is more readily determinable than [Ba], and in which, therefore, such an advantage exists.

The calculations indicated by Equation 22 may be performed graphically with the aid of the curve (Fig. 9) expressing values of U ($U = \dfrac{\beta_M}{0.575} = 1$ for maximum β_M) and of $\log \dfrac{[H^+]}{K_a'}$ (which is equal to $pK_a' - pH$) plotted by L. J. Henderson in the note forming part of this paper. By this curve, when β_M is known, the corresponding value of $pK_a' - pH$, and hence the value of pK_a' may be found by inspection. Likewise, when the pK_a' of an acid buffer is known, the β value at any pH may be found on the curve.

If instead of taking $\dfrac{dB}{dpH}$ as the unit of buffer value we used $\dfrac{dB}{d[H^+]}$, or, to obtain positive values, $\dfrac{dA}{d[H^+]}$ where $A = -B =$ added strong acid, we would obtain

$$\text{(a)} \quad \frac{dA}{d[H^+]} = -\frac{dB}{d[H^+]} = \frac{C K_a'}{(K_a' + [H^+])^2}$$

The molecular buffer value in these terms would be

$$\text{(b)} \quad \frac{dA}{C\,d[H^+]} = \frac{K_a'}{(K_a' + [H^+])^2}$$

The second derivation is

$$\text{(c)} \quad \frac{d^2A}{C\,d[H^+]^2} = -\frac{2 K_a'}{(K_a' + [H^+])^3}$$

From Equation c it is apparent that there is no point of maximum $\dfrac{dA}{d[H^+]}$ value. The second derivative at no point changes through zero from a positive to a negative value or *vice versa*. It is always negative, showing that $\dfrac{dB}{d[H^+]}$ decreases continuously as long as $[H^+]$ has any finite value. For mixtures containing only the free buffer acid and its salt, without additional alkali, minimum $[H^+]$, and therefore maximum $\dfrac{dA}{d[H^+]}$ values, are obtained when all the acid is bound as salt. At this point $\dfrac{dA}{d[H^+]} = \dfrac{1}{K_a'}$. Yet experience has led experimenters to decide that weak acids act most efficiently as buffers when they are present about half as free acid, [Ha], half as salt, [Ba] (Clark,[2] 1920). For buffer mixtures containing equal parts of [Ha] and [Ba], the value of $\dfrac{dA}{d[H^+]}$ is $\dfrac{1}{4 K_a'}$ (obtained by substituting

in Equation b $\dfrac{K_a' \,[Ha]}{[Ba]}$ for the value of [H$^+$] obtained by solving for [H$^+$] Henderson's equation $K_a' = \dfrac{[H^+]\,[Ba]}{[Ha]}$). This means that, with half the buffer acid free in each case, half in the form of its salt, the $\dfrac{dA}{d[H^+]}$ values for the three phosphoric acids, H(H$_2$PO$_4$), H(HKPO$_4$), and H(K$_2$PO$_4$), would be in round numbers, respectively, $\dfrac{1}{4 \times 10^{-2}}, \dfrac{1}{4 \times 10^{-7}}$, and $\dfrac{1}{4 \times 10^{-12}}$, or 25, 2,500,000, and 250,000,000,000.

In the most alkaline of the three solutions, where H(K$_2$PO$_4$) = K$_3$PO$_4$, $\dfrac{dA}{d[H^+]}$ is enormous, merely because [H$^+$] is infinitesimal and d[H$^+$] is proportionally reduced. If we based the unit of buffer value on ability to minimize [OH$'$] instead of [H$^+$] change, that is, on the value of $\dfrac{dB}{d[OH']}$, conditions would be reversed, and the buffer value of the alkaline phosphate mixture would be estimated as a billion times greater than that of the acid phosphate. If, to avoid this embarrassment, we used $\dfrac{dA}{d[H^+]}$ for the acid range, $\dfrac{dB}{d[OH']}$ for the alkaline, the acid and alkaline phosphate mixtures would each have similar buffer values, but 100,000 times greater than the neutral H (KHPO$_4$) − K(KHPO$_4$) mixture. Similar huge ranges of figures are encountered if we use the unit $1 - \dfrac{d[H^+]}{dA}$.

These anomalies and inconveniences disappear when we use the logarithmic $\dfrac{dB}{dpH}$ unit. Each buffer in terms of this unit has the same maximum effect, $\dfrac{dB}{dpH} = 0.575\ C$, when [Ha] = [Ba], and each has within the zone where its effect is exerted, the same symmetrical curve of buffer value, the U-curve of Fig. 9.

For expressing reaction changes, Clark and Lubs (1916) have emphasized the advantages of Sörensen's pH unit, due to its avoidance of huge ranges of numbers, to the elegance with which pH data over wide ranges of reaction may be graphically plotted, to the fact that errors in our methods of determination are proportional to changes in pH rather than in [H$^+$], and to the fact that changes in pH rather than in [H$^+$] appear to be proportional to changes in physiological effects.

For expressing buffer values the pH unit adds to these advantages that of yielding a uniform and symmetrical β curve for buffers, and a uniform maximum value.

The curve[4] of Van Slyke and Zacharias (1914), showing the rate of action of urease in M/11 phosphate at varying pH, affords an illustration of the parallelism between pH change (or proportional [H$^+$] change) and effect on an enzymic activity, and of the relative lack of parallelism between absolute [H$^+$] change and effect on activity. In Table II are indicated the pH and [H$^+$] changes accompanying the same decrease in rate of enzyme action on both sides of the optimum pH. Approximately the same pH change, positive on one side, negative on the other, accompanies the same decrease in rate of enzyme action on both the alkaline and

TABLE II.

	Acid side of optimum.	Alkaline side of optimum.
pH when urease reaction rate = 0.4	6.25	8.47
" " " " " = 0.3....... ..	5 80	8.90
" change...............	−0.45	+0.43
[H$^+$] when reaction rate = 0.4.........	5.6×10^{-7}	0.034×10^{-7}
" " " " = 0.3..............	15.9×10^{-7}	0.012×10^{-7}
" change.............................	$+10\ 3 \times 10^{-7}$	$-0\ 022 \times 10^{-7}$
Mean [H$^+$]................................	$10\ 7 \times 10^{-7}$	0.023×10^{-7}
Proportion by which [H$^+$] is changed = $\dfrac{[\text{H}^+] \text{ change}}{\text{mean } [\text{H}^+]}$	$+0.96$	-0.96

acid sides. It follows that the proportions, + 0.96 and − 0.96, by which [H$^+$] is changed are (in this case exactly) equal. But the absolute [H$^+$] change on the acid side, 10.3×10^{-7} is about 500 times that (0.02×10^{-7}) required on the alkaline side to cause the same decrease in rate of enzyme action. The parallelism between pH change and effect on biological reactions is not, of course, regularly as accurate as happens to be the case in this example. But it appears to be true that rates of enzyme action (and most reactions in the organism appear to be enzymic) as a rule form fairly symmetrical curves when plotted against pH or log [H$^+$], with some degree of quantitative parallelism on both sides of the optimum between pH change and its effect; while there is no such approach to parallelism between effect and absolute [H$^+$] change.

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[4] Van Slyke and Zacharias (1914), p. 194.

General Equation Indicating the Dissociation of a Weak Acid at All Reactions within the Limits of Validity of the Mass Law.—As stated above Henderson's equation is valid only within a range of reaction which is ordinarily 7 ± 3 or 4, and in consequence the equations expressing the values of β that have been derived above by differentiation of Henderson's equation are subject to the same limitation. In order to express the relationships of β to the hydrion concentration of the solution, and the concentration and dissociation constant of the buffer acid, in an equation valid for as great a range of conditions as that limiting the validity of the mass law for dilute acids, we must derive a form of Equation 5 that is general for mixtures of weak acids and their salts, and not limited to the condition that $[H^+]$ and $[OH']$ shall be negligible in comparison with C and $[Ba]$. In order to obtain the desired equation in a form that can be differentiated for calculation of $\dfrac{dB}{dpH}$ it is necessary furthermore to express the values of $[a']$ and $[Ha]$ in terms of C and B.

Let C = total molecular concentration of buffer acid, in form of either free acid or its salt.

    B = concentration of strong base BOH added to acid (gram equivalents of base per liter of solution).

   [Ba] = concentration of salt Ba.

   [Ha] = concentration of undissociated free buffer acid.

   $[a']$ = concentration of anion formed by dissociation of the acid Ha and the salt Ba.

   γ_s = fraction of buffer salt Ba dissociated into B^+ and a' ions.

   γ_b = fraction of free strong base BOH dissociated into B^+ and OH' (BOH is, of course, present only in alkaline solutions such as that of Na_3PO_4).

We wish to ascertain general values of $[A']$ and $[Ha]$ to insert in the basic Equation 5, $K_a [Ha] = [H^+] [a']$. The value of $[a']$ may be ascertained, by equating the concentrations of all the negative and positive ions, respectively.

(23)
$$[B^+] + [H^+] = [a'] + [OH']$$
$$[a'] = [B^+] + [H^+] - [OH']$$
$$= [B^+] + [H^+] - \frac{K_w}{[H^+]}$$

The terms constituting functions of $[H^+]$ in the right-hand member we may use as they are. $[B^+]$, however, we must obtain in terms of the total base added, B, and of $[H^+]$.

(24)
$$[B^+] = \gamma_s [Ba] + \gamma_B [BOH]$$

$$[BOH] = \frac{[OH']}{\gamma_B} = \frac{K_w}{\gamma_B [H^+]}$$

$$[Ba] = B - [BOH]$$

(25)
$$= B - \frac{K_w'}{\gamma_B [H^+]}$$

Substituting in Equation 24 for $[BOH]$ its equivalent as given above, and for $[Ba]$ its equivalent from Equation 25 we have

$$[B^+] = \gamma_s \left(B - \frac{K_w}{\gamma_B [H^+]} \right) + \frac{K_w}{[H^+]}$$

$$= \gamma_s B + \left(1 - \frac{\gamma_s}{\gamma_B} \right) \frac{K_w}{[H^+]}$$

Substituting the above value for $[B^+]$ in Equation 23 we have

$$[a'] = \gamma_s B + \left(1 - \frac{\gamma_s}{\gamma_B} \right) \frac{K_w}{[H^+]} + [H^+] - \frac{K_w}{[H^+]}, \text{ or}$$

(26)
$$[a'] = \gamma_s B + [H^+] - \frac{\gamma_s K_w}{\gamma_B [H^+]}$$

The concentration of undissociated free buffer acid, $[Ha]$, we find by subtracting from the total buffer concentration, C, the part represented by $[Ba]$ in the form of salt, and the part, equal to $[H^+]$, in the form of free acid dissociated according to the equation $[Ha] = [H^+] + [a']$

$$[Ha] = C - [Ba] - [H^+]$$

Substituting for $[Ba]$ its value, as found above in Equation 25, we have

(27)
$$[Ha] = C - B + \frac{K_w}{\gamma_B [H^+]} - [H^+]$$

Substituting in our basic Equation 5 the above values for [a'] and [Ha] gives us

$$(28) \qquad K_a = \frac{[H^+]\left(\gamma_S B - \dfrac{\gamma_S [OH']}{\gamma_B} + [H^+]\right)}{C - B + \dfrac{[OH']}{\gamma_B} - [H^+]}$$

Substituting $K_a' \gamma_S$ for K_a, and dividing both sides of the equation by γ_S, we obtain

$$(29) \qquad K_a' = \frac{[H^+]\left(B - \dfrac{[OH']}{\gamma_B} + \dfrac{[H^+]}{\gamma_S}\right)}{C - B + \dfrac{[OH']}{\gamma_B} - [H^+]}$$

With the introduction of but slight error we may simplify Equation 29 by substituting 1 for γ_B, since in solutions more dilute than 0.01 N, NaOH and KOH approach complete dissociation. We may also in the term $\dfrac{[H^+]}{\gamma_S}$ substitute 1 for γ_S, since in weak buffer acid-salt solutions of such high acidity that $[H^+]$ is an important factor in the equation, the ratio $\dfrac{[Ha]}{[Ba]}$ is sure to be high, with $[Ba]$ correspondingly dilute, and γ_S approaching 1 in consequence. Thus simplified Equation 29 becomes

$$(30) \qquad K_a' = \frac{[H^+](B + [H^+] - [OH'])}{C - (B + [H^+] - [OH'])}$$

In solutions of free acids, $B = O$ and $[OH']$ is negligible. Equation 30 then becomes $K_a' = \dfrac{[H^+]^2}{C - [H^+]}$, which is equivalent to Ostwald's dilution law (Equation 6) since, with only free acid present, $C - [H^+] = [Ha]$. In a solution containing both buffer salts and free acid, and with $[H^+]$ and $[OH']$ both insignificant in comparison with B (solution bordering neutrality), Equation 30 becomes $K_a' = \dfrac{[H^+]B}{C - B}$, which is equivalent to Henderson's equation (Equation 7). Both Ostwald's and Henderson's equations are, therefore, special cases of Equation 30.

In acid and near-neutral solutions (pH <10), [OH'] becomes negligible and Equation 30 becomes

$$(31) \qquad K_a' = \frac{[H^+](B + [H^+])}{C - (B + [H^+])}$$

In alkaline and near-neutral solutions (pH > 4) it becomes

$$(32) \qquad K_a' = \frac{[H^+](B - [OH'])}{C - (B - [OH'])}$$

We may test the approximate accuracy of Equation 30 in the acid and alkaline range by applying it to the titration curves of W. M. Clark[5] (1920), for $H_3PO_4 + KOH$ and for $H(K_2PO_4) + KOH$. (For the neutral range it has already been tested in the form of Henderson's equation in Table I.) Clark titrated 50 cc. of 0.1 M (or 0.3 N) H_3PO_4 with 150 cc. of 0.1 N KOH, and determined the pH curve. The volume was, therefore, increasing throughout the titration. From Clark's curves we obtain the data of Table III. The published curves are drawn on a scale which limits pH readings to an accuracy of about 0.05, but this suffices, considering the wide range covered, to permit use of the data to test the approximate accuracy of Equation 30. As seen from the last 2 columns, the equation gives a true constant.

In order to differentiate Equation 30 we substitute $\dfrac{K_w}{[H^+]}$ for [OH'], and K for the term $[H^+][OH']$, solve the equation for B, and obtain

$$B = \frac{K_a' C [H^+] - K_a' [H^+]^2 - [H^+]^3 + K_w K_a' + K_w [H^+]}{[H^+](K_a' + [H^+])}$$

$$= \frac{[H^+] K_a' C}{[H^+](K_a' + [H^+])} - \frac{[H^+]^2 (K_a' + [H^+])}{[H^+](K_a' + [H^+])} + \frac{K_w (K_a' + [H^+])}{[H^+](K_a' + [H^+])}$$

$$(33) \qquad = \frac{K_a' C}{K_a' + [H^+]} - [H^+] + \frac{K_w}{[H^+]}$$

$$(34) \qquad \frac{dB}{d[H^+]} = - \frac{K_a' C}{(K_a' + [H^+])^2} - 1 - \frac{K_w}{[H^+]^2}$$

$$\frac{dB}{dpH} = - 2.3 [H^+] \frac{dB}{d[H^+]}$$

$$(35) \qquad \beta = \frac{dB}{dpH} = 2.3 \left(\frac{K_a' C [H^+]}{(K_a' + [H^+])^2} + [H^+] + [OH'] \right)$$

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[5] Clark (1920), p. 32.

TABLE III.

Calculation of K'_a Values of H_3PO_4 – KH_2PO_4 and of HK_2PO_4 – K_3PO_4 Mixtures by Means of Equation 30.

Mixtures present.	0.1 N KOH added to 50 cc. 0.1 M H₃PO₄.	V total volume of solution.	$C = \frac{0.1 \times 50}{V}$	$B = \frac{0.1 \times cc.\ KOH}{V}$	pH	[H⁺]	[OH']	K'_a	$pK'_a = -\log K'_a$
	cc.	cc.	N	N		N	N		
H₃PO₄ – KH₂PO₄	0	50	0.1000	0.0000	1.60	0.0251	0.0000	0.84 × 10⁻²*	2.08
	10	60	0.0834	0.0167	1.80	0.0159	0.0000	1.01 "	2.00
	20	70	0.0714	0.0286	2.00	0.0100	0.0000	1.18 "	1.93
	30	80	0.0625	0.0375	2.30	0.0050	0.0000	1.06 "	1.97
	40	90	0.0556	0.0444	2.70	0.0020	0.0000	1.01 "	2.00
Average of last 4 calculations..........								1.07 × 10⁻²	1.97
HK₂PO₄ – K₃PO₄	100 + 10	160	0.0313†	0.0062	11.20	6.31 × 10⁻¹²	0.0016	1.10 × 10⁻¹²	11.96
	100 + 20	170	0.0294	0.0118	11.55	2.82 "	0.0036	1.08 "	11.97
	100 + 30	180	0.0278	0.0168	11.80	1.59 "	0.0063	0.96 "	12.02
	100 + 40	190	0.0263	0.0211	11.95	1.12 "	0.0089	0.97 "	12.01
Average............................								1.03 × 10⁻¹²	11.99

* The first figures, 0.84×10^{-2} and 2.08, really represent K_a rather than K'_a, since there is present only free acid without salt. Since $K_a = \gamma_B K'_a$, if γ_B in the other determinations has a value of 0.8, they would yield an average K'_a value of $0.8 \times 1.08 \times 10^{-2} = 0.85 \times 10^{-2}$.

† The base, [B], is calculated from the amount added in excess of the 100 cc. required to neutralize the first two hydrogens of H_3PO_4.

Equation 35 is identical with Equation 17, which expresses the entire range of buffer values of a solution containing a weak acid plus amounts of strong acid or alkali not limited to equivalence with the weak buffer acid. It is evident therefore, that Equation 35, as an approximation, is valid in general for solutions containing acid buffers of all dissociation constants, at all pH's and concentration ranges, within which the mass action law holds—that is, for buffer concentrations up to about 0.1 M, and a pH range between 2 and 12, and perhaps as wide as between 1 and 13, and for amounts of added strong alkali or acid not limited to any relationship to the amount of buffer acid present.

It is evident from Equation 35 that when $[H^+]$ or $[OH']$ is of significant magnitude in comparison with $\dfrac{K_a' C}{(K_a' + [H^+])^2}$, the buffer effect will be correspondingly greater than that calculated from Equation 12 on the basis of Henderson's equation. Such is the case with the higher acidities attainable with $H_3PO_4 - BH_2PO_4$ mixtures, and with the higher alkalinities attainable with $HB_2PO_4 - B_3PO_4$ mixtures. Thus in Fig. 6 we have in the central part, the curve of $H(KHPO_4) - K(KHPO_4)$ mixtures, typical in shape and symmetry for a buffer acid acting in conformity with Henderson's equation, and with its center at pH $= pK_a'$; while the other two ends curve symmetrically towards horizontal asymptotes. The $H_3PO_4 - KH_2PO_4$ curve however, instead of showing a decreasing slope as the proportion of free H_3PO_4 exceeds $\frac{1}{2}$ molecule, shows a slope constantly increasing to the end. This is because, as shown in Equation 35, the buffer effect of the $[H^+]$ itself, represented by the separate term $[H^+]$ in the equation

$$\beta = 2.3 \left(\frac{K_a' C[H^+]}{K_a' + [H^+]} + [H^+] + [OH'] \right),$$ becomes so great, at the low pH (1.5) reached, that it prevents the decrease in the slope of the pH curve that otherwise occurs as $[Ha]$ approaches C, and in fact causes an increase. A similar increase of slope is observed, because of the high $[OH']$, at the alkaline end of the $HK_2PO_4 - K_3PO_4$ curve.

The quantitative rôles are indicated by Fig. 7 of the buffer effects due to incomplete dissociation of the phosphoric acids, and the buffer effects due to the $[H^+]$ and $[OH']$, respectively, at the more extreme pH limits by the two lower curves in somewhat lighter lines than the

upper curve representing the total effect. The curves are calculated by Equation 35 using K_a' values of 1.97, 6.85, and 12.0, respectively, for the three acids.
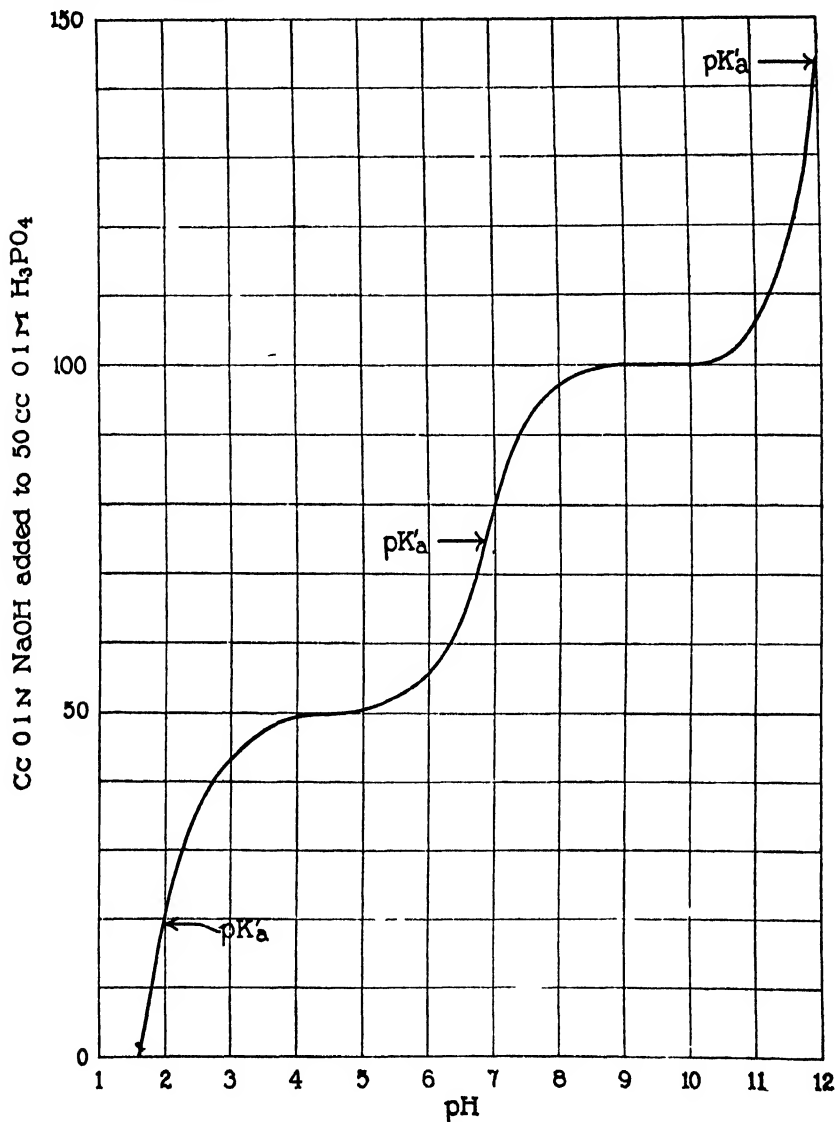
FIG. 6. Clark's titration curve of H_3PO_4.

It will be noted that the β values at the alkaline end of the curve in Fig. 7 are somewhat less than indicated by the slope of this end of the curve in Fig. 6. The

disagreement is not actual, however.   Fig. 7 is calculated on the assumption of a constant volume with C = 0.1 M; while Fig. 6 represents the results of a titration in which the volume was greatly increased at the end.   The disagreement would entirely disappear if the values of Fig. 7 were estimated on the basis of actual C and B values calculated from the varying volume.
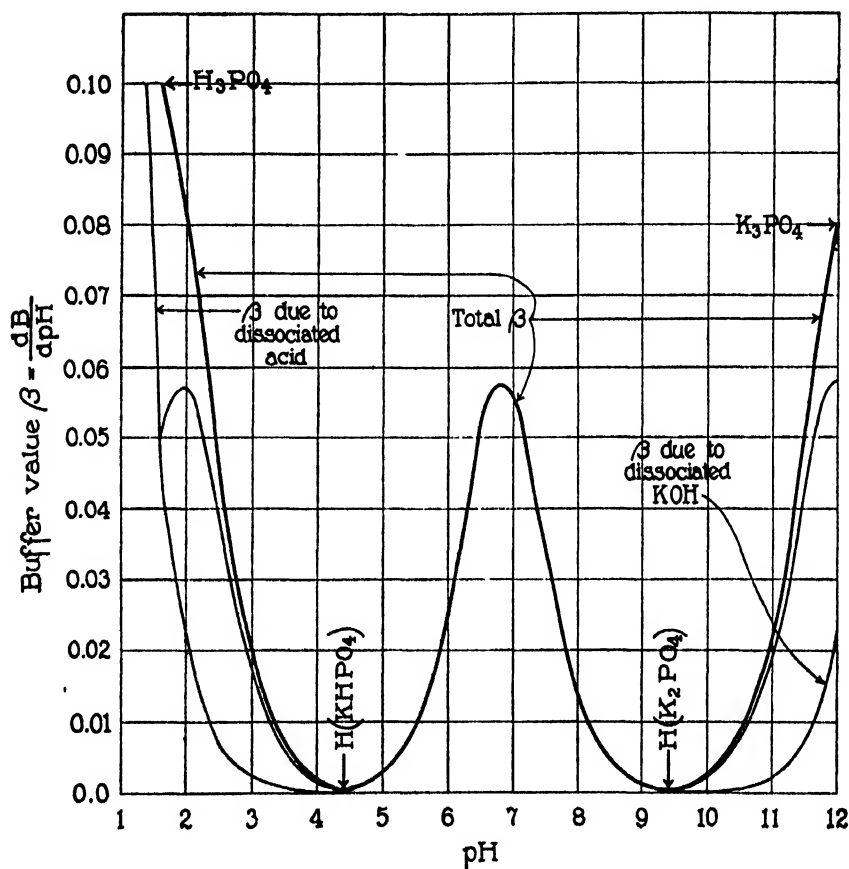
FIG. 7.   Buffer values of the three phosphoric acids.   The total buffer value is indicated by the heavy upper curve, which represents the total β value calculated by Equation 35.

At the more acid and alkaline ranges the partial β values due solely to dissociated free acid and alkali, respectively, become significant, and are indicated by the marked lower curves; while the remaining partial β values, due to repression of [H+] change, are indicated by symmetrical curves similar to that of H(BHPO₄) in the center.

Polyvalent Acid Buffers and Mixtures of Monovalent Acid Buffers.

For a solution containing a mixture of buffers, the buffer effect at any pH is the sum of the separate effects of each buffer in repressing [H+] change, plus the buffer effect peculiar to the [H+] and [OH'].

$$(36) \quad \beta = 2.3\,[H^+]\left(\frac{K'_{a_1}C_1}{(K'_{a_1}+[H^+])^2} + \frac{K'_{a_2}C_2}{(K'_{a_2}+[H^+])^2} + \ldots\right) + 2.3\,([H^+]+[OH'])$$

If $C_1 = C_2 = C_3$, as when the various acid groups belong to the same molecule of a polyvalent acid, Equation 36 simplifies to

$$(37) \quad \beta = 2.3\,[H^+]\,C\left(\frac{K'_{a_1}}{(K'_{a_1}+[H^+])^2} + \frac{K'_{a_2}}{(K'_{a_2}+[H^+])^2} + \ldots\right) + 2.3\,([H^+]+[OH'])$$

In a polyvalent buffer, or a mixture of buffers, the K'_a value and buffer effect β_1, β_2, or β_3, etc., of any one acid or acid group may be determined, under the following conditions.

1. *The pK'_a values of the different acids or acid groups are, as in H_3PO_4, so far apart that the buffer effects do not overlap.* In this case at [H+] values where β_1 is of significant magnitude, β_2 and β_3 are not, and $\beta = \beta_1$, β_2, and β_3 being practically zero.

The extent to which a buffer exerts its effect on both sides of the point pH = pK'_a is roughly indicated by the following table.

pH	Proportion of maximal buffer effect.
	per cent
pK'_a	100.0
$pK'_a \pm 1$	33.0
$pK'_a \pm 2$	4.0
$pK'_a \pm 3$	0.4
$pK'_a \pm 4$	0.04

(The effect is accurately shown for all values within the range pH = $pK'_a \pm 2$ by Fig. 9 in the attached note by Henderson.)

An example of a polyvalent buffer with acid groups whose buffer effects do not overlap is given, as stated above, by H_3PO_4, and shown in Figs. 5 and 6.

2. *The buffer effects partially overlap.* In this case the calculations can be made from the parts of the curve where each buffer is free from the influence of the other, and can be checked by calculations of the total β and B in the pH range where they are summations of the overlapping effects.

3. *The effective range of a buffer is completely overlapped by those of others, of which, however, the K_a' and C values are known.* In this case we may assume for example that two buffers overlap with their buffer effects β_1 and β_2 the effect β_3 of a third buffer. If β_1 and β_2 can be calculated, β_3 can be estimated by subtracting β_1 and β_2 from the total observed buffer value,

$$\beta_3 = \beta - \beta_1 - \beta_2$$

From β_3 thus ascertained one may calculate K_a' by Equation 22. The calculation of β_1 and β_2 from known K_{a1}' and K_{a2}' and of K_{a3}' from β_3, is made most simply, however, graphically, by inspection of the U-curve of Fig. 9.

When the total amount of base, B, is known, a similar procedure can be followed to use it instead of β to determine the dissociation constants. From Equation 33 one determines B_1 and B_2, and by subtracting them from the total base B obtains B_3. This is substituted in Equation 30 to determine K_{a3}'. This procedure is somewhat simpler than that based on β determinations.

An example of the use of the above modes of procedure in determining the separate dissociation constants of a polyvalent acid is contained in a subsequent paper by Hastings and Van Slyke (1922) on citric acid.

It is to be noted that the maximum β_M of a polyvalent buffer may exceed 0.575 if the effects of the different buffer groups overlap. When $\beta = \beta_1 + \beta_2 + \beta_3$, if at the pH where β_2 has its maximum value of 0.575, β_1 and β_3 are of significant size, we shall have $\beta = 0.575 + \beta_1 + \beta_3$. In the case of citric acid, for example, the maximum β_M is 0.84. See also Fig. 8 for demonstration of the summation of buffer effects when the pK' values are nearer together than 3 units.

Basic Buffers.

If we designate the undissociated weak base as bOH (a small b is used to differentiate the weak base from B, the strong base), we have

as our primary equation for dissociation of a weak base, Equation 38, analogous to Equation 5.

$$(38) \qquad K_b = \frac{[OH'][b^+]}{bOH}$$

If we have a mixture of a salt bA and the free base bOH, the equation becomes, analogous to Equation 28

$$(39) \qquad K_b = \frac{[OH'] \left(\gamma_s A - \frac{\gamma_s}{\gamma_A}[H^+] + [OH'] \right)}{C - A + \frac{[H^+]}{\gamma_s} - [OH']}$$

If we let $K_b' = \dfrac{K_{\bar{w}} \, \gamma_s}{K_b}$, substitute in Equation 39 $\dfrac{K_w \, \gamma_s}{K_b}$ for K_b, $\dfrac{K_w}{[H^+]}$ for $[OH']$, and $- B$ for A, and differentiate B with respect to $[H^+]$, as in obtaining Equation 35, we obtain an equation (Equation 40) identical with Equation 35, except that K_b' replaces K_a'.

$$(40) \qquad \frac{dB}{dpH} = 2.3 \left(\frac{K_b' \, C \, [H^+]}{(K_b' + [H^+])^2} + [H^+] + [OH'] \right)$$

It follows, therefore, that all the equations and relationships that have been demonstrated to hold for acid buffers hold likewise for basic ones, the only difference being that in equations covering the behavior of the latter the approximate constant K_b', which equals $\dfrac{K_w \, \gamma_s}{K_b}$, replaces the approximate constant K_a' which equals $\dfrac{K_a}{\gamma_s}$, of the equations covering the behavior of acid buffers.

Amphoteric Buffers.

In the case of an amphoteric buffer with acid groups, having K_a' values K_{a1}', K_{a2}', etc., and basic groups having K_b' values K_{b1}', K_{b2}', etc., we have, by summation

$$(41) \qquad \beta = 2.3 \, C \, [H^+] \left(\frac{K_{a1}'}{(K_{a1}' + [H^+])^2} + \frac{K_{a2}'}{(K_{a2}' + [H^+])^2} \cdots + \right.$$
$$\left. \frac{K_{b1}'}{(K_w + K_{b1}'[H^+])^2} + \frac{K_{b2}'}{(K_w + K_{b2}'[H^+])^2} \right) + 2.3 \, ([H^+] + [OH'])$$

The discussion under "Polyvalent· acid buffers" applies also to amphoteric buffers, practically without modification except that pK_b' is to be used in place of pK_a' for the basic group.

Universal Buffer Mixtures.

Acree and his coworkers (1921) have pointed out that "there are advantages to be derived from the use of buffer mixtures covering a wide pH range . . . . . and so selected as to form a practically continuous, as well as smooth curve," in other words, to maintain a constant buffer value. From the considerations of the present paper it appears possible to predict the relationships of the buffers that will form such a mixture. Approximate constancy of buffer value will be maintained in a mixture of total buffer value

$$\beta = \beta_1 + \beta_2 + \beta_3 \, . \, . \, . \, .$$

under the following conditions. Each buffer must have its pK' at such a distance from its adjacent neighbor in the series that the overlapping buffer effects at $pH = pK_1'$ equal those at $pH = \dfrac{pK_1' + pK_2'}{2}$, or at a pH midway between two pK' values. Under these conditions the total buffer value midway between the maximum of a given buffer and that of the adjacent buffer will be the same as the buffer values at the maxima, and the fluctuations between can be but slight. The solution of the problem is approximated by calculating the total buffer values by means of Equation 12. The calculation is most readily done graphically by means of Fig. 9. Over most of the range covered in the calculations of Table II, it was necessary to add 5 buffer values at the point $pH = pK_1'$, and 6 at the point $pH = \dfrac{pK_1' + pK_2'}{2}$, midway between 2 pK"s.

It is apparent from Table IV that the ideal universal buffer would be approximated by a mixture of buffers forming a series with their pK' values separated by 1 unit, each K' being 10-fold as great as the next lower. In a solution containing such a series of buffers in equivalent concentration the total buffer value varies less than 1 per cent. When the intervals between pK"s are 1.4, the total buffer

value shows a difference of about 10 per cent between maxima and minima. When the difference is 2.0 the maximum buffer values exceed the minimum by over 50 per cent.

When the pK′ intervals are regular, the most constant total buffer value is obtainable by using all buffers in the same concentration. In case, however, some of the intervals are longer than the rest so that one or two buffers are more isolated from the rest and consequently less supported by overlapping buffer effects of their neighbors, such buffers are to be used in sufficiently greater concentration to bring the total buffer value in these zones of activity up to that of the rest of the system.

TABLE IV.

Interval between pK_1' and pK_2' . . . etc.	Total buffer value, calculated as sum of molecular buffer values of individual buffers.	
i	at $pH = pK'$	at $pH = pK' + \frac{i}{2}$
3.0	0.577	0.138
2.0	0.598	0.384
1.6	0.684	0.552
1.4	0.749	0.673
1.3	0.784	0.738
1.2	0.848	0.813
1.1	0.919	0.899
1.0	1.003	0.998

Fig. 8 shows graphically the total buffer values obtained by equimolecular mixtures of buffer series, of which the pK′ intervals are 1, 2, and 3, respectively.

The Buffer Value of Blood.

From the $BHCO_3$: pH curve for normal arterial blood under varying CO_2 tensions given by the writer in a previous article (1921, b, Fig. 2) it is possible to calculate the buffer value. At the reaction range pH 7.0 to 7.8, dB = dBa, since the amount of B in the form of BOH is negligible. The increase in $BHCO_3$ with increasing CO_2 tension and falling pH is equal to the decrease in Ba of the other buffers, since the base used to form additional $BHCO_3$ comes from

FIG. 8.

544
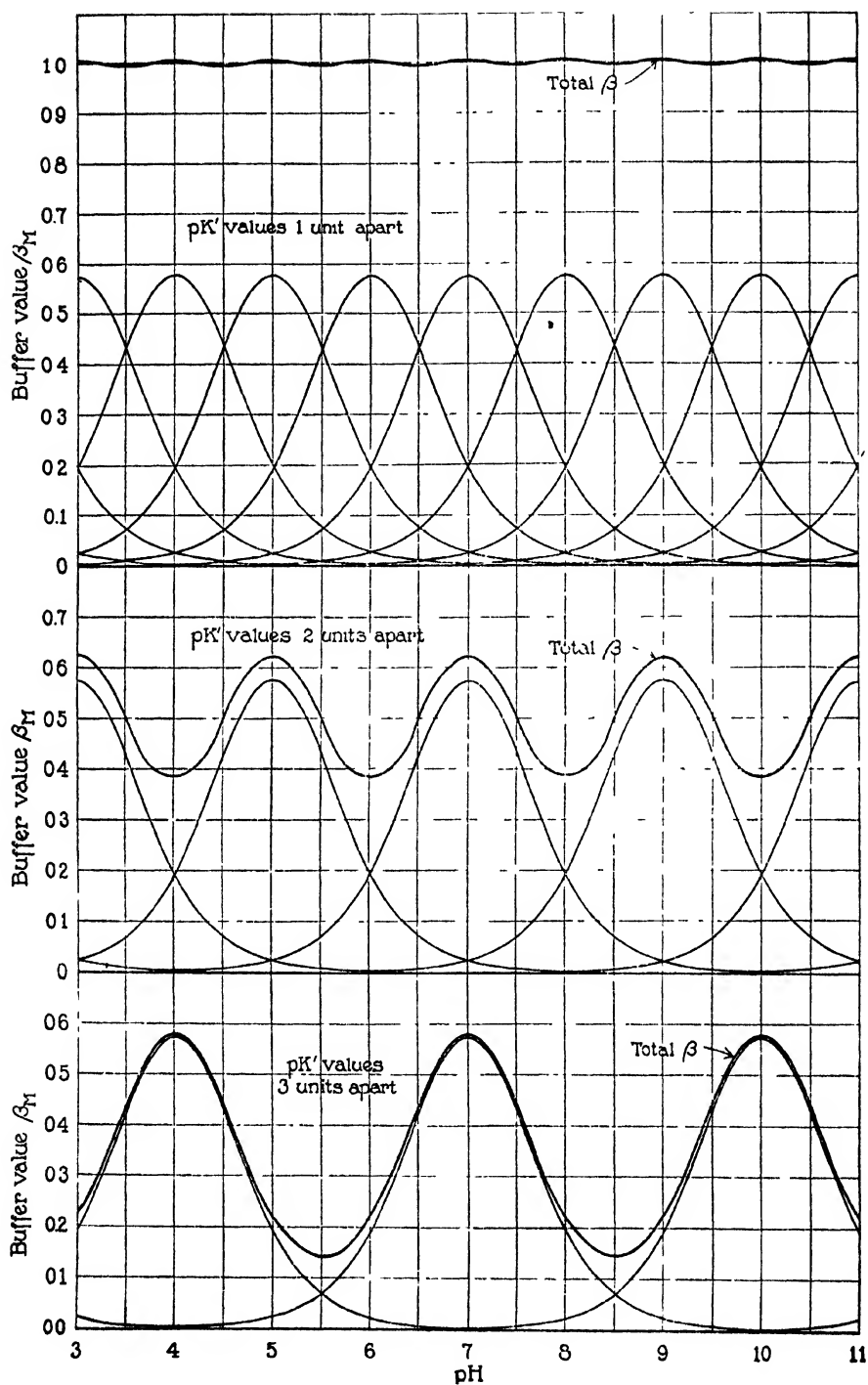
them. Consequently, for the buffers other than BHCO$_3$, $\dfrac{dB}{dpH}$ =

$\dfrac{- \, dBHCO_3}{dpH}$. Between pH 7.0 and 7.8 (mean pH = 7.4; ΔpH = 0.8) the mean molecular concentration of BHCO$_3$ decreases from 0.0285 to 0.0120 (Δ Ba = 0.0165). Therefore, at mean pH 7.4, for the buffers other than BHCO$_3$, $\beta = \dfrac{\Delta Ba}{\Delta pH} = \dfrac{0.0165}{0.8} = 0.0206$.

The buffers other than BHCO$_3$ are chiefly proteins. In fact, they appear to be chiefly the weak acid groups in the hemoglobin (Van Slyke, 1921, a).[6] If these groups were exerting their maximum buffer efficiency, having their pK$'$ values near 7.4, their total concentration would be C $= \dfrac{\beta}{0.575} = \dfrac{0.0206}{0.575} = 0.036$ N (Equation 20), and they would be combined with 0.5 equivalent of alkali, or 0.018 N, since, at pH = pK$'$, Ba $= \dfrac{C}{2}$. If pK$'$ were less than 7.4, then Ba would exceed 0.018 N; if pK$'$ were more than 7.4, then Ba, the base bound to protein, would be less than 0.018 N. As a matter of fact, as closely as one can estimate from the only data available (Greenwald, 1922), 0.018 N is about the concentration of alkali bound to proteins in the blood. If this is substantiated (experiments are in progress), it means that in normal blood the active buffer groups other than those of BHCO$_3$, total about 0.036 N in concentration. The hemoglobin concentration is about 0.008 M, if one assumes that 1 molecule of hemoglobin binds 1 molecule of oxygen. Probably, therefore, $\dfrac{3.6}{0.8} = 4.5$ represents more or less approximately the number of COOH groups in each oxyhemoglobin molecule that are active at blood pH.

In Greenwald's method the proteins are precipitated with an excess of picric acid and the picric acid combined with alkali is determined as the difference between total picrate and free titratable picric acid. Essentially this procedure removes the protein and total H$_2$CO$_3$, and measures the picric acid required to

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[6] Van Slyke (1921, a,) p. 160.

neutralize the bases that were combined with them. In order to measure this value exactly in such a procedure the titration of the free picric acid would require the pH of blood for its end-point (Van Slyke, Stillman, and Cullen, 1919).[7] Greenwald used two end-points, one (methyl red) 2 pH units on the acid side of 7.4, the other (thymolphthalein) about equally on the alkaline side. The average normal concentrations of base thus measured were 0.0435 and 0.0315 N, respectively, with the two indicators, the mean value, 0.0375 N being probably close to the amount of base bound by H_2CO_3 and protein in normal blood. If we subtract from 0.0375 N the 0.0205 N base normally bound as bicarbonate (Van Slyke, 1921, b),[8] we have left 0.0170 N base bound by the proteins.

If to the buffer value of the buffers other than the bicarbonate we add that of bicarbonate we obtain the total buffer value of the blood. The value for the $BHCO_3$ and H_2CO_3 buffer at pH 7.4 may be calculated from Equation 12. At pH 7.4, $[H^+] = 4 \times 10^{-8}$; C = $[BHCO_3]$ + $[H_2CO_3]$ = 0.0215; and $K'_a = 8 \times 10^{-7}$ (Henderson and Haggard). Substituting these numerical values in Equation 12 we have

$$\frac{dB}{dpH} = 2.3 \frac{4 \times 10^{-8} \times 0.0215 \times 8 \times 10^{-7}}{(8 \times 10^{-7} + 4 \times 10^{-8})^2} = 0.0022$$

Adding 0.0022 to 0.0206, the β of the other buffers, gives $\beta = 0.0228$ as the average total buffer value of normal human blood at pH 7.4.

The total *concentration* of buffers active in the blood at pH 7.4 is at least 0.036 N (buffers other than bicarbonate) + 0.021 N ($BHCO_3$ + H_2CO_3) = 0.057 N. The alkali bound by these buffers, which are apparently all of the weak acid type, appears to be about 0.04 N (0.038 N bound by protein and H_2CO_3 (Greenwald, 1922), and traces by other buffers).

The above estimates of buffer concentration and buffer alkali are merely first approximations. The 0.04 N buffer alkali figure is a rough estimate. The 0.057 N total buffer concentration may have to be increased in proportion as the active protein buffer groups are found to have pK' values differing from 7.4. The figure 0.022 for the average $\frac{dB}{dpH}$ value of human blood is based on fairly complete observations and is probably about correct.

[7] Van Slyke, Stillman, and Cullen (1919), p. 167.
[8] Van Slyke (1921, b), p. 170.

The ability of the blood *in vivo* to neutralize *non-volatile acids without change in pH* is not due to buffer action, that is to the incomplete dissociation of the weak buffer acids set free by reactions such as $HCl + Ba = Ha + BCl$. It is due to the volatility of one of these acids, CO_2, and the ability of the respiratory apparatus to remove the excess CO_2 set free by such a reaction as $HCl + BHCO_3 = BCl + H_2CO_3$. Because CO_2 set free is thus removed, the bicarbonate is, as shown by the writer (1921, *a*), the most important form of the blood's alkali reserve available ·for neutralizing non-volatile acids, such as β-hydroxybutyric.

For neutralization of added H_2CO_3, however, (in venous blood) the $BHCO_3$ formed can exert only its actual buffer effect, which is about 0.1 that of the other blood buffers. For preservation of the blood's neutrality against retention of non-volatile acids the bicarbonate, because of the volatility of the CO_2 set free from it, is the most important single constituent; while for preservation of neutrality against CO_2 retention, hemoglobin, because of its buffer power, is the most important constituent.

Determination of the Buffer Values of a Solution by Titration.

The experimental determination of the buffer value, β, of a given solution at a given pH may be performed in either of the following two ways.

1. *By determination of the slope of the B, pH curve at the given pH.* One adds strong standard acid or alkali in varying amounts to the solution, and determines the pH after each addition. If the concentration of added standard acid or alkali be great enough to keep the maximum increase in volume of the buffer solution relatively small, *e.g.* below 50 per cent of the original, the volume change may ordinarily be neglected, as the pH values of most buffer solutions are but slightly affected by volume changes of such magnitude. Addition of each cc. of N NaOH to a buffer solution of original volume V cc. causes an increase of $\Delta B = \dfrac{1}{V}$. The buffer value, $\dfrac{dB}{dpH}$, at any pH may be determined graphically by drawing a line tangent to the curve at that pH and measuring the slope of the line. (See Fig. 1 and its· discussion.) (The tangent can be drawn most accurately by a de-

vice employed by engineers and called to my attention by Dr. Northrop. A mirror is laid at right angles across the curve, so that the curve on paper is continuous with its reflection. A line drawn across *the curve along the edge of the mirror is then* perpendicular to the tangent, which with the help of a square is laid off at right angles to the mark of the mirror-edge.)

2. *By measuring the amount of strong acid or alkali required to cause a pH change over a measured range.* One merely adds as in the above procedure, a measured amount of standard alkali or acid, and determines the pH before and after the addition. If the added alkali is

<center>TABLE V.</center>

$\dfrac{[Bs]}{C}$ or $\dfrac{bOH}{C}$	$\dfrac{[Ha]}{C}$ or $\dfrac{bA}{C}$	$\dfrac{[H^+]}{K}$	$\log\dfrac{[H^+]}{K} = pK' - pH$	$0.4343\,\beta_M = V$	$\dfrac{dV}{d\log[H^+]}$	$\dfrac{\beta}{0.575} = U$		
0.01	0.99	99.0	1.996	0.0099	−0.0097	0 0396		
0.02	0.98	49.0	1.690	0.0196	−0.0192	0.0784		
0.05	0.95	19 0	1.279	0.0475	−0.0428	0.1900		
0.10	0.90	9 0	0.954	0.0900	−0 0720	0.3600		
0.15	0.85	5.67	0.753	0.1275	−0.0893	0.5100		
0.20	0.80	4.00	0.602	0.1600	−0.0960	0.6400		
0.25	0.75	3.00	0.477	0.1875	−0.0938	0.7500		
0.30	0.70	2.33	0.368	0.2100	−0.0840	0.8400		
0.35	0.65	1.858	0.269	0.2275	−0.0683	0.9100		
0.40	0.60	1 500	0.176	0.2400	−0.0480	0.9600		
0.45	0.55	1.222	0.087	0.2475	−0.0248	0.9900		
0.50	0.50	1.000	0.000	0.2500	0.0000	1.0000	50 per cent	75 per cent
0.55	0.45	0.818	0.913−1	0.2475	0 0248	0.9900		
0.60	0.40	0.667	0.824−1	0.2400	0.0480	0.9600		
0.65	0.35	0.538	0.731−1	0.2275	0.0683	0.9100		
0.70	0.30	0.429	0.632−1	0.2100	0.0840	0.8400		
0.75	0.25	0.333	0.523−1	0.1875	0.0938	0.7500		
0.80	0.20	0.250	0.398−1	0.1600	0.0960	0.6400		
0.85	0.15	0.176	0.247−1	0.1275	0.0893	0.5100		
0.90	0.10	0.111	0.046−1	0.0900	0.0720	0.3600		
0.95	0.05	0.0526	0.721−2	0.0475	0.0428	0.1900		
0.98	0.02	0.0204	0.310−2	0.0196	0.0192	0.0784		
0.99	0.01	0.0101	0.004−2	0.0099	0.0097	0.0396		

Δ B, and the increase in pH is Δ pH, then the *average* buffer value between the two pH's observed is $\beta = \dfrac{\Delta B}{\Delta pH}$. For example, if 10 cc. of M NaOH are added to 1 liter of a buffer solution of unknown value, $\Delta B = \dfrac{10 \times 1}{1,000} = 0.01$. If the pH values before and after the additions are 7 and 8.5, respectively, $\Delta pH = 8.5 - 7 = 1.5, \beta = \dfrac{0.01}{1.5} = 0.0067$. 0.0067 represents the average value of β between pH 7 and 8.5, or approximately the β at pH 7.75. Table I gives an example of this manner of determining values of β.

If the average β value over a certain pH range is used as an approximation of the exact β value at the mean pH, as in Table I, it is essential that the pH intervals be not too great. Where the B, pH curve is nearly straight a pH range exceeding 1 may be used to determine β at the mean pH, as in the case of citric acid; but where there is much curvature the range should be less in order to yield accurate mean values.

Note by Lawrence J. Henderson.

Professor Henderson consented to criticize the preliminary draft of the above paper and suggested that the relationships involved in the first and second differential derivatives of his equation (Equation 7) might be more readily perceived as outlined by the following series of equations, and by Table V and Fig. 9. The latter demonstrate over the main significant range, the interrelation between: (1) buffer effect, (2) ratio of buffer dissociation constant to hydrion concentration of the solution, (3) ratio of buffer salt to free buffer base or acid, and (4) value of the second differential.

First Derivative.

Given a buffer mixture such that

$$[Ha] + [Ba] = C$$

If

$$[H^+] = K\frac{[Ha]}{[Ba]}$$

$$[H^+] = K\frac{[Ha]}{C - [Ha]}$$

$$[H^+] C = [H^+][Ha] + K[Ha]$$

$$[Ha] = \frac{[H^+]C}{K + [H^+]}$$

FIG. 9.

550
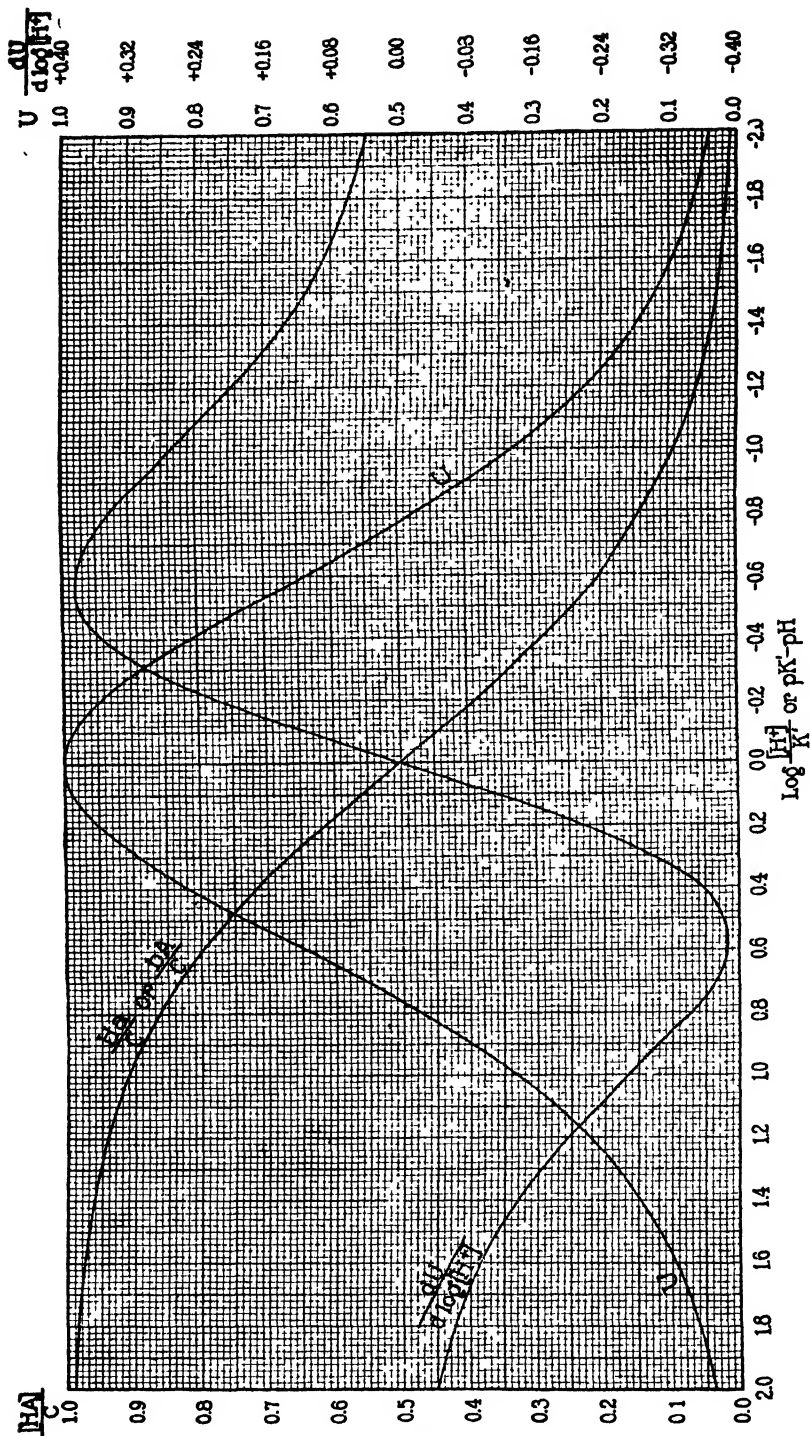
Imagine addition of strong acid or strong base

$$\frac{d\,[Ha]}{d\,[H^+]} = \frac{(K + [H^+])\,C - [H^+]\,C}{(K + [H^+])^2} = \frac{C}{K + [H^+]} - \frac{C}{K + [H^+]} \times \frac{[H^+]}{K + [H^+]}$$

$$\frac{d\,[Ha]}{d\,[H^+]} = \frac{C}{K + [H^+]}\left(1 - \frac{[H^+]}{K + [H^+]}\right) = \frac{C}{K + [H^+]} \times \frac{K}{K + [H^+]}$$

$$\beta_M \times \log_e 10 = V = \frac{d\,[Ha]}{d\,[H^+]} \times \frac{d\,[H^+]}{d\log\,[H^+]} \times \frac{1}{C} = \frac{d\,[Ha]}{d\log\,[H^+]} \times \frac{1}{C} = \frac{[H^+]}{K + [H^+]} \times \frac{K}{K + [H^+]}$$

$$V = \frac{1}{1 + \dfrac{[H^+]}{K}} \times \frac{1}{1 + \dfrac{K}{[H^+]}} \quad \ldots \ldots \ldots \ldots \quad (1)$$

But
$$\frac{[Ha]}{C} = \frac{[H^+]}{K + [H^+]} = \frac{1}{1 + \dfrac{K}{[H^+]}}$$

And
$$\frac{[Ba]}{C} = \frac{1}{1 + \dfrac{[H^+]}{K}}$$

Hence, alternatively, leaving out the constant factor $\dfrac{1}{\log_e 10}$,

$$\begin{cases} \beta_M = \dfrac{[Ha]}{C} \times \dfrac{[Ba]}{C} \\[2mm] \beta_M = \dfrac{[Ha]}{C} - \dfrac{[Ha]^2}{C^2} \\[2mm] \beta_M = \dfrac{[Ba]}{C} - \dfrac{[Ba]^2}{C^2} \end{cases}$$

Second Derivative.

$$\beta_M \times \log_e 10 = V = \frac{H}{K + [H^+]} \times \frac{K}{K + [H^+]}$$

$$\frac{dV}{d[H^+]} = \frac{K}{K + [H^+]} \times \frac{(K + [H^+]) - [H^+]}{(K + [H^+])^2} + \frac{[H^+]}{K + [H^+]} \times \frac{-K}{(K + [H^+])^2}$$

$$\frac{dV}{d\log[H^+]} = \frac{dV}{d[H^+]} \times \frac{d[H^+]}{d\log[H^+]} = \frac{K}{K + [H^+]} \times \frac{K}{K + [H^+]} \times \frac{[H^+]}{K + [H^+]} -$$
$$\frac{K}{K + [H^+]} \times \frac{[H^+]}{K + [H^+]} \times \frac{[H^+]}{K + [H^+]}$$

$$\frac{dV}{d\log[H^+]} = V\left(\frac{1}{1 + \dfrac{[H^+]}{K}} - \frac{1}{1 + \dfrac{K}{[H^+]}}\right) \quad \ldots \ldots \ldots \ldots \ldots (2)$$

Hence, alternatively, leaving out the constant factor $\dfrac{1}{(\log_e 10)^2}$

$$\frac{dV}{d\log[H^+]} = \frac{[Ha]}{C} \times \frac{[Ba]}{C} \times \left(\frac{[Ba]}{C} - \frac{[Ha]}{C}\right)$$

SUMMARY.

As a numerical measure of the buffer value of a solution the number of gram equivalents of strong alkali or acid taken up per unit change in pH has been used. In these terms, each cc. of N alkali or acid that must be added to a liter of solution to raise or lower its pH by 1 adds 0.001 to the buffer value.

Since the buffer value varies with varying pH, the value at any given pH is defined by the ratio $\dfrac{dB}{dpH}$.

A general form of the mass action equations, $K = \dfrac{[H^+][a']}{[Ha]}$ and $K = \dfrac{[OH'][b']}{[bOH]}$, covering the dissocations of weak acids and bases, respectively, in the presence of their salts, has been developed and differentiated. The general equation obtained expressing the buffer value, β, is

$$\beta = \frac{dB}{dpH} = 2.3 \left(\frac{K'[H^+]C}{(K'+[H^+])^2} + [H^+] + [OH'] \right)$$

$K' = \dfrac{K_a}{\gamma_s}$ for weak acids, where K_a is the acid dissociation constant, γ_s the fraction of the salt Ba dissociated into $[B^+]$ and $[a']$ ions. For weak bases $K' = \dfrac{K_w \gamma_s}{K_b}$. C indicates the concentration of the buffer. When $C=0$, and no weak acid or base is present, the buffer equation simplifies to $\beta = 2.3 ([H^+] + [OH'])$, which indicates the buffer value of water plus strong acid or alkali.

When pH is within the limits of about 3 and 11, and C has a value not much less than 0.1 N, $[H^+]$ and $[OH']$ are relatively so small that the buffer equation simplifies to

$$\beta = 2.3 \frac{K'[H^+]C}{(K'+[H^+])^2}$$

Under these conditions the molecular buffer value $\beta_M = \dfrac{\beta}{C} = 2.3 \dfrac{K'[H^+]}{(K'+[H^+])^2}$. β has its maximum value for every buffer when

$[H^+] = K'$.   At this point $\beta_{M} = \dfrac{2.3}{4} = 0.575$ for every buffer, and one-half the buffer is in the form of free acid or base, one-half in the form of its salt.   That is, maximum buffer value is exerted when $\dfrac{[H^+]}{K'} = \dfrac{Ba}{Ha}\left(\text{or} = \dfrac{bOH}{bA}\right) = 1$.   For given values of these ratios greater or less than 1, definite proportions of the maximum buffer effect are exerted (Fig. 9).

Applications of the above facts are indicated, including the determination of the acid and basic dissociation constants and the molecular weights of buffers, and the calculation of the buffer values of solutions of mixed, polyvalent, and amphoteric buffers.   The technique for experimental determination of the buffer values of solutions is outlined.

The buffer value of normal blood at pH 7.4 is shown to approximate that of a 0.04 N solution of a buffer acid with $K' = 10^{-7.4}$

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Note Added to Proof.—Koppel and Spiro (1914), in a paper overlooked until the above went to press, have utilized a similar, though not identical buffer unit, *vis.* $\dfrac{d(S - S_0)}{dpH}$, *where S_0 represents the amount of strong acid that would produce* the change dpH if added to an unbuffered solution of a given initial pH, while S represents the actual amount of strong acid required in the buffered solution to cause the same pH change. Within the pH range of validity of Henderson's equation this unit becomes numerically identical with ours (except for being its negative value), since S_0 is negligible; and Koppel and Spiro have reached some conclusions identical with ours for solutions within this range, *vis.*, concerning the occurrence of a constant, maximum, buffer value when $K_a = [H^+]$. For pH values outside the range of validity of Henderson's equation, Koppel and Spiro's unit is not identical with the, we believe, more simply applied $\dfrac{dB}{dpH}$ unit developed in this paper.

HERPETOMONAS MUSCÆ DOMESTICÆ, ITS BEHAVIOR AND EFFECT IN LABORATORY ANIMALS.

By R. W. GLASER.

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PLATE 11.

While making some observations on the habits of blood sucking flies, at a large dairy during the summer of 1921, the writer noticed that many of the house flies frequenting cattle were engorged with blood. A microscopic examination of the alimentary tract proved that the blood was obtained from cows. Closer observations revealed the fact that house flies frequently feed at the punctures recently deserted by *Stomoxys calcitrans* or *Haematobia serrata*. It was noticed that house flies hovered near stable or horn flies when feeding and immediately pounced upon the wounds inflicted by these insects as soon as the biting flies had flown away to rest and digest their meals. The house flies in turn sucked blood until engorged. These observations demonstrated certain possibilities under which house flies might act as transmitters of microörganisms and parasites found at times within the peripheral circulation.

Darling in 1912 proved that *Musca domestica* was able to transmit *Trypanosoma hippicum* to healthy mules after feeding on the sores of diseased ones.

Patton and Cragg in 1913 showed that some non-biting members of the genus Musca, like *M. pattoni*, associated themselves around cattle with such biting forms as Haematopota, Stomoxys, Philaematomyia, and other forms. Most of the non-biting species feed only on blood and depend entirely on the biting species for the preparation of their food. Patton and Cragg further noticed that the non-biters were not house flies, but species which are seldom found away from cattle. In the Philippines in 1912, Mitzmain observed a close association between non-biting flies and Stomoxys, but the non-biting species were not identified.

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Since no protozoa were at this time found in biting flies, and since the house fly offered possibilities for the dissemination of blood parasites, it became important to make a careful study of the protozoan fauna found in the alimentary tract of such flies; to attempt to cultivate these protozoa, and determine their pathogenic properties. Three species of flagellates were found in the digestive tract of house flies during the summer of 1921, but only one of these will receive consideration at this time.

Herpetomonas muscae-domesticae Burnett was by far the most prevalent flagellate inhabiting the digestive tract of adult house flies. When the Herpetomonad was present at all, it was nearly always there in enormous numbers; sometimes the entire intestinal contents seemed to consist of little else. In order to avoid falling into an error, the writer considered only those flies parasitised which showed the true flagellated form. Possible preflagellate or postflagellate types of the parasite were not tabulated for the reason that some of these are easily confused with certain stages of the two other species obtained and since cultivated by me in pure culture.

Dissections of house flies from June 1 until the middle of November gave the following percentages of infected individuals.

	Number dissected.	Infection.
June	80	12.5 per cent.
July	110	41.0 per cent.
August	125	52.0 per cent.
September	95	21.0 per cent.
October	50	4.0 per cent.
November	50	0 per cent.

Judging from the writings of others, the precentages of infected flies found here in New Jersey correspond more nearly to the percentages obtained by workers in warmer climates. In Madras, Patton found 100 per cent. infected. In Syria, Wenyon states that the majority of the house flies harbored *H. muscae-domesticae.* Prowazek found 8 per cent. infection at Rovigno, while Franchini and Mantovani found a 3 per cent. infection near Boulogne. Dunkerly and Hewitt were unable to find the parasite in England and the writer knows of no records in America.

Franchini and Mantovani (1915) discovered their infected flies on farms in the environment of Boulogne. Flies caught in the houses

of Boulogne showed no Herpetomonads. This observation was independently verified by the writer during the summer. In going over his notes he does not find a single record of a dissection of a fly caught within dwelling houses that revealed any flagellates whatever. All of the parasitised flies were caught in cow barns and stables. This seems to suggest a rather close association between the Herpetomonad and domestic animals.

The flagellate under discussion received much attention by other workers on account of its similarity to the parasite of Kala Azar. Prowazek (1904), Patton (1908–1909), Porter (1909), MacKinnon (1910), Wenyon (1911 and 1913) fully described the life history and cytology of the flagellate from films and sections of the intestines of house flies. During all of this time much discussion arose concerning various morphological details. Prowazek, Lingard and Jennings, claimed that the Herpetomonad had a double flagellum, while Leger Patton, Porter, MacKinnon, and Wenyon insisted that the animal had a single flagellum and that a double flagellum represents the beginning of longitudinal division of the parasite.

The present writer obtained some excellent films from flies fixed and stained with Giemsa which seem to prove that the flagellum is single and that the so-called "double nature" of the flagellum is a division phenomenon. Figures, 1, 2, 3 and 4, show various types of the mature and maturing Herpetomonad. The trophonucleus is situated near the center of the lancet shaped body. The blepharoplast is located at the anterior flagellar end. Figure 5 represents the flagellum in the act of splitting longitudinally accompanied or preceded by the division of the blepharoplast. Figure 6 shows, a further advance consisting in the division of the trophonucleus accompanied or succeeded by cell division. A separation of the two halves of the flagellum is noticed at the anterior end of the protozoan. Figure 7 is the last stage preceding the separation of the two daughter halves. Figure 3 shows a deeply staining thread which runs backward from the kinetonucleus. That this thread is spirally coiled as claimed by Prowazek could not be determined. In the writer's specimens the thread appears nearly straight. Some individuals were found which showed a distinct vacuole at the extreme anterior end (Fig. 3). Figures 8 and 9 represent other morphological forms of the same species of Herpetomonad fre-

quently encountered.   A cytopharynx as found by Wenyon (1913) was not observed.   Moreover, whole or even parts of bacteria are not found within the endoplasm.   It is highly probable that the protozoan is nourished through osmotic diffusion currents.

Since the morphology and life history of the flagellate has been completely worked out by others, the writer attempted very little in this direction.   In anticipation of experimental work, however, it was thought important to identify the species here in New Jersey with the European form.   Notwithstanding various difficulties encountered in interpreting conflicting statements in the literature concerning the morphology of *Herpetomonas muscae-domesticae* (often also referred to as *Leptomonas muscae-domesticae* or *Crithidia muscae-domesticae*), the writer is fairly certain that he is dealing if not with the identical species, at least with a variety very closely related to the form or forms studied by Prowazek, Patton, Wenyon, Franchini, Mantovani, and other European and Asiatic workers.

In 1915, a fresh impetus was imparted to the whole Herpetomonad work by Laveran and Franchini.   These investigators found a Herpetomonad in the mouse flea resembling *H. muscae-domesticae* in many respects.   They were able to cultivate the flagellate in pure culture on the Nicolle, Novy, and McNeal medium.   Laveran and Franchini found Leishmaniform bodies in their cultures after a few days which later developed into true Herpetomonads.   Later, in 1919, these same observers produced a typical form of Leishmaniasis in white mice by inoculating them with cultures of the Herpetomonad found in the intestine of the mouse flea.   Rats also proved susceptible and developed Leishmaniasis, better called Herpetomaniasis as suggested by Fantham and Porter (1916).   The disease was also reproduced by feeding the parasites.   The Herpetomonads of *Anopheles maculipennis* and *Melophagus ovinus* produced similar conditions.   In 1919, Chatton also cultivated a Herpetomonad from the dog flea and reproduced Leishmaniasis in dogs.

In all of these experimental cases, the animals were inoculated or fed with the flagellated forms found in culture.   After periods varying between six weeks and two and a half months, the animals began to show symptoms such as fever, emaciation, with loss of appetite and weight.   Often Leishmaniform and sometimes flagellated forms were

found in the blood. Autopsies of very sick animals, and those that died of the disease usually showed an enlargement of the spleen in which the rounded or oval Leishmaniform bodies were found.[*] These bodies also proved quite numerous in the bone marrow. The liver rarely revealed any parasites and the other organs never.

In 1916, Fantham and Porter reported a large number of experiments with Herpetomonads and Crithidia obtained from the digestive tract of a variety of insects. With these flagellates they were able to produce Herpetomoniasis in mice, dogs, canaries, sparrows, martins, grass snakes, lizards, frogs, toads, and sticklebacks. From the experiments the authors concluded that:

"Herpetomoniasis or Leishmaniases can be induced in various warm and cold blooded vertebrates when the latter are inoculated or fed with Herpetomonads occurring in the digestive tracts of various insects. The infection produced and the protozoan parasites found in the vertebrates resemble those of human and canine Leishmaniases." "The disease induced may run an acute or a chronic course. In the acute cases among the vertebrates, the flagellate form of the parasite was the more obvious at death. In chronic cases, non-flagellate forms of the parasite were more numerous."

Fantham and Porter argue that since the flagellated stages of *Leishmania donovani* and *L. tropica* are now known, the links completing the evidence that a Leishmania is a Herpetomonas are complete. They further express the belief "that Leishmaniases are invertebrate-borne Herpetomoniases, and that these maladies have been evolved from flagellates of invertebrates (especially Herpetomonads of insects) which have been able to adapt themselves to life in vertebrates."

In 1915, Franchini and Mantovani stated that they were able to produce Leishmaniasis or Herpetomoniasis in rats with *H. muscae-domesticae*, the house fly parasite. Besides reproducing the usual symptoms of Herpetomoniasis and the Leishmaniform stages in the involved organs, they were further able to cultivate the organism indirectly. House flies in contradistinction to fleas and some other insects have a prolific intestinal flora which makes it impossible to obtain pure cultures directly from any part of the alimentary canal. The present writer has many times convinced himself of the fruitlessness of such an attempt. The bacteria carried over into the media

together with the *Herpetomonads* soon outgrow them and in a short time kill off the flagellates. These protozoa seem to be able to tolerate a certain amount of intestinal flora under intestinal conditions, but soon die out under artificial conditions, i. e., on artificial media upon which bacteria multiply so rapidly.

Franchini and Mantovani took blood from the heart of one of their rats inoculated three and one-half months previously with the intestinal contents of parasitised house flies. Some of this blood was inoculated into the condensation liquid of the N. N. N. medium (1 part) mixed with a 3 per cent. solution of glucose (4 parts). In about twelve days a pure culture of little organisms appeared having the aspect of ana-plasms in stained smears. At autopsy two mice inoculated intraperi-toneally with this culture showed a few Leishmaniform parasites in the liver. The authors were unable to propagate their cultures in trans-plants.

The present writer inoculated intraperitoneally four white and two wild mice, one rat, and one guinea pig with the intestinal contents of flies heavily parasitised with *Herpetomonas muscae-domesticae*. Care was taken to obtain parasites in the flagellated condition and they seemed active and vigorous. The inoculated animals showed no clinical symptoms whatever. Their blood was examined in stained and unstained condition at intervals of a few days, but nothing was found. Two of the white mice were autopsied in one month. All of the organs were normal and no Leishmaniform bodies were visible in the liver, spleen, bone marrow, kidneys, or other organs. One white mouse and one wild mouse were killed after two months. The examin-ations were entirely negative. One white mouse, one wild mouse, and the rat were sacrificed in three months. The white mouse had pneu-monic lungs, but in every other respect the three animals were normal. No parasites were found. Since the examinations of the sup-posedly susceptible animals proved negative, and since the guinea pig showed no symptoms nor parasites in the blood, it was not killed and is still alive today, five months after the inoculation. At no time during this period did any temperature develop nor was there any loss in weight.

The negative results obtained by the writer need not reflect on any of the results obtained by Franchini and Mantovani, and suggest that

the authors may have dealt with a geographical variety or with a distinct species, although for morphological reasons the European form and the form studied from this locality may be considered identical. It may be suggested that the *Herpetomonads* of house flies in different parts of the world be carefully compared, and also that a careful seasonal study of the forms occurring in one region be made.

Since it seemed impossible to obtain a pure culture of *H. muscae-domesticae* free from bacteria indirectly by the inoculation of higher animals, another method that proved successful was devised. In 1918, while experimenting with grasshopper diseases and strains of d'Herelle's *Coccobacillus acridiorum,** it was found that many species of Acridians developed an immunity toward bacteria. Many Acridians die of bacterial infections annually, but many also recover and become sexually mature. Such recovered hoppers are comparatively immune and this immunity can be demonstrated. Experimentally also, it was shown that this immunity could be produced in healthy non-exposed animals by inoculation with sublethal doses or with killed cultures of various bacteria. The idea suggested itself that it might be possible to inoculate grasshoppers or Locustids with the intestinal contents of flies containing Herpetomonads and bacteria, and perhaps obtain a pure culture of the protozoa in this way.

In the first experiment, fifty large female grasshoppers (*Melanoplus femur-rubrum*) were inoculated with such material. On August 3 the intestines of five heavily parasitised house flies were removed under aseptic conditions and cut up very fine in sterile Locke's solution in order to liberate the Herpetomonads and mince the intestine, so that no large pieces of tissue would be introduced into the hoppers. The hoppers were then held and restrained, so that the inoculation site could be wiped off with alcohol corrosive sublimate mixture. Twenty-five hoppers were then inoculated each with 0.1 c.c. of Locke's solution containing Herpetomonads, bacteria, intestinal cells and contents. Ten hoppers were inoculated into the body cavity on the ventral side between the thorax and abdomen, and fifteen were inoculated in the hind leg joint between the trochanter and femur. Twenty-five uninoculated hoppers were kept as controls. The inoculated and

* A systematic study of the organisms distributed under the name of *Coccobacillus acridiorum*. d'Herelle. Annals Ent. Soc. America, 1918, vol. xi.

uninoculated hoppers were then placed into separate sterile glass jars with some grass.

In 48 hours all but four of the inoculated hoppers were dead. The uninoculated ones were all alive. The dead hoppers gave off an odor of putrefaction and were swarming with bacteria. No Herpetomonads were seen. The four inoculated, but live hoppers were also carefully examined. The leg joint between the trochanter and femur of the hind leg was first wiped off with alcohol and then singed. A sharp, sterile capillary pipette was then introduced and some blood removed. Some of this blood was introduced into ordinary culture media to test for bacterial sterility. The rest was examined microscopically for bacteria and Herpetomonads. In none of the four hoppers were bacteria found microscopically, but in three of the animals a few actively moving Herpetomonads were seen. These preparations were stained by Giemsa's method and the flagellates were identical with those introduced. The N.N.N. medium and a variety of other media were inoculated with some of the blood containing Herpetomonads, but no growth was obtained at room or incubator temperature although tubes were kept for two weeks and examined every few days. Some tubes were also sealed in order to produce a lowered oxygen tension. The media previously inoculated with blood to prove bacterial sterility remained sterile.

Blood from the control hoppers was carefully examined for flagellates in exactly the same manner. No bacteria nor protozoa of any sort were found. In the writer's previous experiences with hundreds of hoppers comprising many species, he has never found protozoa in the blood of these animals.

These experiments, therefore, prove that out of a large series of hoppers inoculated with the intestinal contents of parasitised house flies, a small number will survive, will free themselves of the intestinal bacteria in about 48 hours, and will maintain *Herpetomonas muscae-domesticae* for at least 48 hours or more.

Since no growth of Herpetomonads was obtained on the media used, an insect medium suggested itself, but since one can secure so little blood serum or tissue juices from hoppers another insect was used.

The writer was fortunately rearing large numbers of the larvae of the meal moth (*Euphestia küniella*). About two hundred large meal

worms were taken and rubbed up in a mortar until nearly all of the juices were expressed. Twenty-five c.c. of sterile Lock's solution was added at intervals to facilitate the grinding. This material was then strained through a cheese cloth after which it was filtered through paper. The filtrate amounted to 35 c.c. This was then filtered through sterile Berkefeld candles. At the same time some fresh horse serum was filtered through a Berkefeld candle. To 10 c.c. of the diluted insect juices, 30 c.c. of horse serum was added and the two materials mixed. This mixture was then tubed into small tubes, 1.5 c.c. per tube. The tubes were then put into an inspissator and the temperature raised to 74 C., at which temperature the serum coagulated. The time during which the temperature was raised from 60 C. to 74 C., and lowered again to 60 C. consumed one hour and thirty minutes. The tubes were then removed. Two drops of normal grasshopper blood were then permitted to flow over each slant, after which the tubes were incubated for 48 hours as a test for bacterial sterility. Later the tubes were stored in the refrigerator after being sealed with sealing wax to prevent evaporation.

August 18, fifteen grasshoppers (*Melanoplus femur-rubrum*) and two locustids (*Amblycorypha oblongifolia*) were again inoculated as in the previous experiment. Fifteen hoppers constituted the controls. In 48 hours all the controls were alive excepting two. These two were examined but nothing excepting bacteria found. All the experimental hoppers but one were dead. The two locustids were alive. Blood was examined from the hopper and two locustids and Herpetomonads were found in all three. No bacteria were present, nor did any growth appear subsequently in inoculated media. Some blood from the three positive cases was inoculated into some of the special insect-horse serum medium previously described. Two tubes were inoculated from each animal, sealed with sealing wax and kept at room temperature. At the end of seven days the tubes were examined and a light growth of Herpetomonads was found in the liquid in the bottom of some of the tubes. The growth was found in one tube inoculated with hopper blood and in two tubes inoculated from the blood of one of the locustids. The two tubes inoculated with blood from the other locustid showed no growth. All the tubes were sterile for bacteria. In the tubes in which growth occurred the Herpetomonads were all in the flagellated

condition growing in rather dense clumps. No pre- or post-flagellated phases were seen and those present, as stained smears demonstrated, appeared to have been produced by the longitudinal division of pre-existing flagellated forms. When the flagellates were examined in Locke's solution they appeared to be quite vigorous and active. although their activity was somewhat hampered by excessively long and wavy flagella. The flagella at times appeared to be twice the length of the flagella seen in preparations of the Herpetomonads direct from fly intestines. Otherwise, the morphology of the cultivated forms corresponded to the long, lancet shaped forms found in flies. Transfers to fresh insect media were immediately made. In the original tubes the flagellates died out during the course of the next week. After a week the transfers again showed a growth, but not nearly so prolific as in the original tubes. Some material from one of these tubes was inoculated intraperitoneally into two white mice. One mouse was autopsied in four weeks and the other in ten weeks. Absolutely nothing was found.

A second cultural transfer was made, but nothing grew. Examinations every few days revealed a partial recovery of the flagellates put in, but these soon died out and no multiplication occurred. The organisms in the first transfer tubes also died out very soon.

While the examinations were being made, 15 hoppers were inoculated September 5. The results were almost identical with the foregoing. A fairly luxuriant growth was obtained on the first set of tubes. Transfers were made and the resulting growth was weaker. Second transfers were again attempted, but no growth ensued.

SUMMARY.

House flies in proximity to cattle were found engorged with cow blood. A close association between the feeding habits of Stomoxys, Hæmatobia and *Musca domestica* was observed. House flies often feed at the punctures deserted by the biting flies. *Herpetomonas muscae-domesticae* was found to be the most prevalent flagellate inhabiting the digestive tract of adult house flies in summer. The number of flies parasitised was large. The greatest degree of parasitism was reached in July and August. The parasitised flies were always caught in cow barns and in horse stables. Flies caught in dwelling houses were not parasitised.

Some morphological details pertaining to the flagellate are discussed and the opinion is expressed that the morphology described is identical with that studied by other workers.

Experimental Leishmaniases or Herpetomoniasis is reviewed and discussed. The writer was unable to produce either with *Herpetomonas muscae-domesticae*.

The view is tendered that probably more than one variety of *Herpetomonas muscae-domesticae* exist and that these varieties may be detected solely on the basis of pathogenic and other physiologic properties.

A special method for the pure cultivation of *Herpetomonas muscae-domesticae* is described. The flagellated form was cultivated and reproduced itself by longitudinal division.

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EXPLANATION OF PLATE 11.

All figures represent *Herpetomonas muscae-domesticae.*   ×813.

Figs. 1–4.—Types of mature and maturing flagellates.

Fig. 3.—Herpetomonad showing deeply stained rod, and vacuole at anterior end.

Fig. 5.—Division of flagellum and blepharoplast.

Fig. 6.—Division of trophonucleus and cell.

Fig. 7.—Advanced cell division.

Figs. 8 and 9.—Other morphological types.

(Glaser: *Herpetomonas muscæ domesticæ.*)

A STUDY OF TRYPANOSOMA AMERICANUM.

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PLATES 14 to 17.

On using some cattle blood for other work, the writer encountered the very large haemoflagellate first described by Crawley in 1909 under the name of *Trypanosoma americanum*. Later in 1912, Crawley more fully described the morphological, cytological and cultural characters of this interesting form. Since the appearance of Crawley's publications much work has been done on Herpetomonas, Crithidia, Leishmania, Trypanosoma, and other genera. By the application of the methods of Novy, the latter investigator and his students, and workers like Laveran, Franchini, Prowazek, Nöller, Wenyon, Kofoid, Fantham, Porter, McCulloch and others, paved the way for a scientific understanding of the true relationships of forms which ontogenetically and phylogenetically had seemed rather doubtfully interested in one another.

It therefore seemed to the writer that a restudy of Crawley's large species might contribute some facts not so easily obtained from smaller forms. *Trypanosoma americanum* is not only a very large species, but it is easily obtained and grown. It occurs around Washington, as Crawley found, in the blood of 74 per cent. of the cattle. The present writer's results on the incidence of infection in New Jersey are not comparable with the total percentage given by Crawley, for the reason that the former's work was begun after the first of October, 1921, and Crawley showed that: "By October the trypanosomes have become much less abundant in the blood, and an animal negative in that month might readily have been positive in July or August." The other purpose of the present investigation was to disclose, if possible, a few facts bearing on the parasitic or pathogenic possibilities of *T. americanum* and the manner of transmission from cow to cow.

. During the months of October, November and December, cow blood was drawn under sterile conditions from the jugular vein at intervals.

Following the cultural directions of Crawley, tubes were prepared containing 6 cc. of the defibrinated cow blood to be tested, above which was placed 6 cc. of sterile bouillon. The tubes were incubated at room temperature and the upper layer of erythrocytes examined for trypanosomes at the end of 3 or 4 days.

The accompanying table shows the number of cases that proved positive for trypanosomes.

Cultural Requirements.—As Crawley showed, the trypanosomes are so rare numerically in cattle that the preparation of cultures offers the only safe method for determining their presence. However, the present

Table Showing Incidence of Infection in Cows and Yearling Heifers.

Date 1921.	Cow.	Results.	Date 1921.	Cow.	Results.
Oct. 15	584	+ +	Nov. 8	Calf 938	− −
Oct. 15	600	− −	Nov. 10	735	− −
Oct. 15	601	+ +	Nov. 10	722	− −
Oct. 25	584	+ +	Nov. 10	757	− −
Oct. 25	600	− −	Nov. 10	896	− −
Oct. 25	601	+ +	Nov. 10	758	− −
Nov. 8	3750	− −	Dec. 12	1096	− −
Nov. 8	843	+ +	Dec. 12	1092	− −
Nov. 8	45	− −	Dec. 12	1085	− −
Nov. 8	3877	− −	Dec. 12	1091	− −
Nov. 8	859	− −	Dec. 12	1075	− −
Nov. 8	3881A	+ +	Dec. 12	1064	− −
Nov. 8	878	+ +	Dec. 12	1063	− −
Nov. 8 .	8858	− −	Dec. 12	1077	− −

Six cc. defibrinated blood were used for each culture. Two cultures were prepared from each sample of blood.

writer centrifuged 50 cc. of fresh defibrinated blood from cow 584. The blood from this cow had previously yielded cultures of trypanosomes. After centrifuging, the bottom layer was examined in a fresh state on several slides with a low power objective. After a long search one *T. americanum* was found. This specimen was very large and morphologically similar to the forms found in early cultures, 12 to 48 hours. The undulating membrane was short and ended anterior to the center of the body. No other specimens were found although others were doubtless present for the same blood from which the samples for centrifuging were removed yielded cultures later.

The cultural requirements and characters of *T. americanum* in liquid media were fully described by Crawley. The writer may add that he had just as good success with horse blood as when cow blood was used. However, after 5 or 6 days the trypanosomes begin to hemolyze the top layer of erythrocytes and it becomes difficult to see the colonies at the junction of the red cells and bouillon. This phenomenon does not appear to occur so rapidly with the cow blood cultures.* They remain clear for quite a long time and consequently one can see well enough to fish out individual colonies with a fine pipette.

The N. N. N. solid medium prepared either with cow or horseblood also proved highly satisfactory. As will be shown later, forms are obtained on this medium not found in liquid cultures. Moreover, the trypanosomes grow very rapidly on the N. N. N. medium during the first 4 or 5 days, and consequently it is a favorable source for the demonstration of dividing individuals. In applying it, however, care must be taken to keep the surface moist by adding sterile horse or cow serum every few days or by placing the petri dishes in a humid atmosphere. Colonies do not develop on the N. N. N. medium; the growth is diffuse. In order to obtain the best results all the cultures should be kept at room temperature; the higher incubator temperatures, 35 to 37 C. inhibit growth and produce degeneration and death in 3 to 5 days.

Cytology of the Stages Found in Cultures.

In the study of this phase of the subject preparations were made from the various cultures at intervals. Some of these preparations were studied in the fresh condition or with intravital dyes. Others were fixed and stained. Crawley dried his films, staining them later with Wright's stain. The writer repeated this procedure, but abandoned it in favor of the "wet method," for the reason that much better and more beautiful preparations resulted. As a general routine, the films were drawn out very thin and then immersed, while still wet, for 15 minutes in half and half absolute alcohol-ether mixture. They were then stained by Novy's modification of the Romanowsky method. This gave by far the best results, although certain cytologic structures

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* It is important to use fresh blood for culture media.

could be better elucidated by using Schaudinn's fixative and Heiden-hain's iron hematoxylin. Mallory's chloride of iron hematoxylin method was also used to advantage in a few cases.

The cultures are best studied 3 days after incubation at room temperature. At this time a sufficient number of trypanosomes have multiplied so that one has little difficulty in finding them. In 3 days the bodies of *T. americanum* averaged 17 to 18μ long. The free part of the flagellum was usually as long as the body or slightly longer. Often enormous forms were encountered. Some of these measured 25μ long or longer, exclusive of the flagellum.

In trypanosomes taken from liquid cow blood cultures 3 days old (Figs.1, 2, 3, and 4), the body is long, more or less cylindrical and rather flexible. The flagellum on reaching the anterior part of the body becomes an undulating membrane which follows the length of the body laterally for about one-third or less of the distance. It then loses itself in the body of the trypanosome ending (Figs. 1 and 2) near a dark staining granule, the parabasal body. No blepharoplast or thickening of the end of the thread itself can be detected. The nucleus when demonstrated, is located centrally (Fig. 1) or anterior to the central (Fig. 2). A karyosome is usually clearly differentiated. The end of the undulating membrane may be some distance from the nucleus (Fig.1) or the two may lie closer together (Fig. 2). In many specimens large and deeply staining granules are distributed through-out the finely granulated cytoplasm. When these granules lie in close proximity to the end of the undulating membrane, it is difficult to determine which one constitutes the parabasal body. However, specimens such as the one represented in figure 2 are frequently found. In these cases only one large deeply staining body is found in the cytoplasm; it is in close proximity to the end of the thread and is consequently interpreted as the parabasal body.* Cases were found (Figs. 3 and 4) in which no nucleus was visible (unless one interprets the large deeply staining granules near the center of the body as such). These cases are difficult to interpret because they were present on slides containing other trypanosomes with well fixed and differentiated · nuclei.

* The nomenclature of Kofoid, Swezy and McCulloch is followed in describing the extranuclear organelles.

In specimens found in 8 day liquid cow blood cultures (Figs. 5 to 9), nearly all individuals have lost the undulating membrane. Many of the organisms have approached the typical herpetomonad type. Figure 5 represents such a case; no undulating membrane, rigid body with the nucleus in an extreme anterior position. Figures 6 and 7 represent bulbous forms with flagella; figures 8 and 9 more extreme types with that organ lacking. In figure 9 nuclear division seems to have occurred. (The writer doubts whether this is a futile attempt at endogenous budding.) Figure 10 represents one of several organisms found in an 8 day horse blood culture showing that some of the bulbous forms are still capable of reproduction.

Forms found in 14 day old cow blood cultures (Figs. 11 to 19) show bulbous types with flagella, and round or oblong ones without that organ and not simulating flagellates in the least. The discovery of such forms as those shown in figures 15 to 18 aroused the writer's suspicions that he may be confronted with the result of a kind of endogenous budding similar to that found by McCulloch (1919) in *Crithidia emryophthalmi*. A. diligent hunt revealed other stages such as that represented by figure 20 from 14 day old cow blood culture, and figure 21, found in a 20 day old cow blood culture. These stages are very rare and the writer has studied his cultures enough to feel sure that they do not develop, but abort. These structures seem to hint at either a phylogenetic recapitulation, or at certain latent developmental possibilities, and if any stages in the invertebrate host exist and were known a further expression of opinion might be possible.

In 20 day old cow blood cultures many degenerating forms appear. After 20 days the cultures gradually die out although a few forms may persist for a much longer time. Figures 22 and 23 represent two unusual forms seen in a 20 day old cow blood culture. In figure 22 a short undulating membrane exists ending definitely at a well defined parabasal body which is here situated alongside of the nucleus. In figure 23 the nuclear system seems to have migrated posteriorly. On the N. N. N. medium with either cow or horse blood development is much more rapid and consequently degeneration occurs much sooner. The life of the trypanosomes on the solid medium is not nearly so long as is the case when it is grown in the blood bouillon mixture.

On 4 day old cultures grown on the N. N. N. plates such forms as those on Plate III appear. Figures 24 and 25 show types similar to those found in early liquid cultures with the exception that the organisms are smaller and the body more attenuated. Figures 26 and 27 represent respectively a large and a small herpetomonad type. Due to the rapid multiplication on the N. N. N. medium one readily obtains many division stages. Figure 28 shows nuclear and cell division, but the parabasal body is still intact. Figure 29 shows another form undergoing nuclear division. Figure 30 demonstrates nuclear, parabasal body, and cell division. Figure 31 represents another form of division superficially simulating constriction. There is no evidence for any other common method of division excepting longitudinal, therefore what happened here was partial longitudinal division accompanied by a movement of the two halves in opposite direction before final separation occurred. Figure 32 shows what appears like an abnormality, cell division without any apparent preceding nuclear division. Figure 33 represents a group of trypanosomes including actively growing and dividing forms, and senile and degenerating individuals. Figures 34 and 35 represent still other conditions encountered.

An examination of the N. N. N. plates in 7 days shows many curious shapes and forms that can only be interpreted as degeneration stages. In 9 days the degeneration stages are well accentuated and the protozoa have a tendency to become very large and amoeboid as shown in fixed and stained preparations. No amoeboid activity can, however, be determined in fresh films examined at room temperature or on the warm stage. Plate IV shows a series of degeneration forms. The appearance is strikingly like that of an amoeba. The cells are large with reticulated cytoplasm containing often many deeply staining granules. Figure 36 still possesses its flagellum, a telltale brand of the organism's flagellate origin. The nucleus has a well defined karyosome and chromatin granules within the clear nuclear substance. Figures 37 and 38 also show large nuclei filled with chromatic material, and figure 39 shows a karyosome as well. Figure 40 shows a long attenuated form. The cells although senile are still in some ways physiologically functional. The nuclei, as has been shown, are normal and the organisms are still at times capable of reproduction (Fig. 41). After about 12

to 14 days, however, the protoplasm of the organisms becomes coarsely granular, Brownian movement is seen within, and complete disintegration soon follows.

Specificity.

Since *Trypanosoma americanum* occurs as a parasite in American cattle in which it produces no visible pathologic conditions, little hope was experienced that the inoculation of laboratory animals would throw any light on the nature of this parasitism. Nevertheless, two guinea pigs, one rabbit, six white and two wild mice were inoculated with young, 3 to 4 day old, cultures. Intraperitoneal inoculation was practiced on the guinea pigs, intravenous on the rabbit and subcutaneous, introperitoneal and intravenous inoculation on the mice. Blood smears were made from time to time from all animals with negative results. Temperatures and weights demonstrated nothing.

One guinea pig was autopsied in 3 weeks. All the organs were carefully examined and cultures made with negative results. The mice, which were given from $\frac{1}{4}$ to 2 cc. of the cultures, dependent on the manner of inoculation, were all autopsied at intervals from 4 days to 2 months. Smears and cultures were made from the peripheral and heart blood, the spleen, liver, kidneys, and bone marrow, but no trypanosomes or stages of trypanosomes were found in smears or recovered in cultures. These results, taken in conjunction with the observations already published on cattle by Crawley, seem to indicate that *T. americanum* is specific to cattle.

It appeared to the writer that an invertebrate host, viz., some insect, might act as the transmitter of *T. americanum*. The insect fauna occurring around the cattle that harbored trypanosomes was carefully studied and only three forms appeared worthy of consideration, namely, *Stomoxys calcitrans, Haematobia serrata*, and *Musca domestica*. It may seem unnecessary to include the latter species, but it has been shown that the house fly will often "follow up" the bites of the true bloodsucking forms and under these conditions will engorge, so that consequently it may be a source of infection and transmission. Moreover, Darling (1912) showed that the house fly is capable of transmitting *T. hippicum* to healthy mules.

Collections of the three flies were made from the bodies and stables of animals known to harbor *T. americanum*. These flies were carefully dissected and the entire alimentary canal including the crop, salivary glands, and Malpighian tubes was examined. Ninety-two house flies, 147 stable flies and 85 horn flies were thus investigated, but no trypanosomes were found. *Herpetomonas muscae-domesticae* was frequently encountered in house flies, but no other protozoa were seen in the two blood suckers. These negative results should not discourage the point of view, still held, that one of these three flies will prove to be responsible for the transmission of *T. americanum*.

In order to determine whether *T. americanum* could survive in flies, 20 Stomoxys and 10 Haematobias were fed with a vigorous liquid culture of this flagellate. Two Stomoxys and two Haematobias were dissected immediately after engorgement and trypanosomes were found in abundance, showing that the flies actually ingested the organisms. Four Stomoxys and four Haematobias were dissected in 24 hours. The flagellates were found in the intestines of all, but had experienced a considerable modification. Many herpetomonad types were seen; rigid, attenuated with no undulating membrane. In 48 hours four more Stomoxys, and the remaining Haematobias were examined. In two cases what appeared like a few degenerating flagellates were seen. All of the remaining Stomoxys were dissected in 72 hours and nothing was found. The flies during this time were well cared for and supplied twice daily with defibrinated horse blood which they ate. This experiment seems to show that Stomoxys and Haematobia might act as the transmitters of *T. americanum*, but that the transfer from host to host must occur within 48 hours. The natural mode of transmission remains problematical, however, until *T. americanum* has actually been found in the intestine or some other organ of a fly caught wild.

Modifying Influence of the Environment, and Taxonomic Position.

Recently Nöller (1920) has cast doubt on the validity of the genus Crithidia and places this genus in synonymy with Trypanosoma. This investigator worked with crithidia-like organisms morphologically very similar to *Trypanosoma americanum*. Nöller showed that trypanosomes could be changed into Crithidia and vice versa. He subjected

the sheep trypanosomes, growing on sheep blood agar, to different temperatures. At 30 C. the organism grew as a Crithidium with a short undulating membrane. At 37 C., in a few days, the body became snake-like, the rigid hind end became flexible and intermediate stages between Crithidia and true blood trypanosomes began to appear.

In the case of the trypanosomes of birds the same phenomenon occurred. At room temperature the organisms grew as Crithidia, but when subjected to 37 C. for 48 to 72 hours true trypanosomes appeared, although multiplication ceased at that temperature. If the plates were returned to room temperature rapid miltiplication resumed in 12 to 48 hours, and the organisms changed again into Crithidia. Nöller also states that Trautmann succeeded with difficulty in converting the crithidial phases of *T. theileri* (an organism parasitic in European cattle) into trypanosomes by subjecting them to a temperature of 37 C. for from 5 to 8 days. From these experiments Nöller concludes that the genus Crithidia is a synonym for Trypanosoma.

Since *T. americanum* is morphologically really a Crithidia the writer repeated Nöller's experiments with this form. The experiments are given in detail below.

, 1. *Trypanosoma americanum* was grown on cow blood agar (2 plates) at room temperature 3 days. At this time an examination showed many active Crithidia with anterior nuclear complex and short undulating membranes. The plates were then placed in incubator at 37 C. and examined in 2 days. Multiplicative energy had ceased and among Crithidia many deformed individuals were found, such as bulbous and round forms. No trypanosomes were seen. In 4 days the organisms were found dead and disintegrating with the exception of a few greatly deformed individuals. This experiment was repeated at 35 C. with a similar result. No trypanosome types ensued.

2. *Trypanosoma americanum* was grown in liquid cow blood bouillon mixture (4 tubes) at room temperature for 3 days. At this time an examination showed active Crithidia with the anterior nuclear complex and short undulating membranes. The tubes were then placed in the incubator at 37 C. One day later the cultures were examined. Multiplication was inhibited. The organisms were crithidia-like with anterior nuclear system and short undulating membranes. The cultures were again examined, in 2, 3, 4, and 5 days, but no conversion into true trypanosomes occurred. Deformed and degenerating individuals began to appear and in 6 to 7 days the cultures had "died out."

This experiment was repeated at 35 C. with a similar result. No trypanosome types ensued.

It might be well to state that all of these cultures were not only studied in the fresh state, but all results were checked with fixed stained preparations.

The above experiments prove that by the method employed the crithidia-like *T. americanum* was not converted into a true trypanosome.

The writer has never seen *T. americanum* approach the trypanosome type under any conditions. In cultures, as was shown above, herpetomonad types are common. These types were also observed, as was shown with the fly experiment, if the crithidial forms are introduced into the invertebrate intestine. Moreover, the examination of very early cultures made from cattle blood always revealed the crithidial type as both Crawley and the writer found. Lastly, the experiment with the freshly drawn and centrifuged blood mentioned in the beginning is also proof of the fact that the crithidia-like phases of *T. americanum* obtained in cultures resemble the forms as they occur within cattle.

It would seem then that the organism under discussion is a Crithidia and not a trypanosome. For morphological reasons this would seem to be the case. In the trypanosomes, the extranuclear organelles are at one time in the natural life cycle, or on some medium, or at some temperature according to Nöller, located posteriorly to the nucleus. Such forms have a well developed undulating membrane. These characters are common to trypanosomes found in the blood. *T. americanum* occurs naturally in the blood, yet the blood forms do not possess these characters nor can they be experimentally induced to assume them. In common with most Crithidia the nucleus is usually centrally located or anteriorly to the center. The extranuclear complex is located anteriorly to the nucleus and a short undulating membrane exists.

In spite of the crithidial nature of *T. americanum*, the writer feels it best not to include this flagellate in the genus Crithidia. The true Crithidia are forms inhabiting the alimentary tract of invertebrates, often in the intestines of plant-feeding insects. Such flagellates have never had occasion to come in contact with vertebrate blood and it would be impossible to predict what might happen to them should this occur. Since the organism under discussion resembles a Crithidium morphologically, but naturally lives in the blood of cattle it seems best to regard it as an intermediate evolutionary stage between the true crithidians and true trypanosomes. For this reason, and on account of

the fact that Crawley's organism has remained in the genus Trypanosoma so long, the writer prefers not to alter its taxonomic status.

In conclusion I wish to state that I am indebted to Dr. Ralph B. Little of this department for collecting the samples of blood.

SUMMARY.

Trypanosoma americanum was successfully grown in horse blood medium and on the N.N.N. medium, as well as in cow blood medium. Development in the culture media was traced, and the cytological details of the various stages described.

T. americanum is specific to cattle. In freshly drawn blood and in very early cultures *T. americanum* resembles the majority of the forms found in 3 and 4 day old cultures.

Morphological and experimental data are presented to show that *T. americanum* is structurally a Crithidium. Prolonged culture and environmental alterations have a tendency to produce herpetomonad types but never trypanosome types.

Reasons are presented in support of the view that *T. americanum* is an intermediate evolutionary stage between true Crithidia and true trypanosomes. The name *Trypanosoma americanum* is retained.

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EXPLANATION OF PLATES.

PLATE 14.

All figures represent *Trypanosoma americanum*. ×813.

Figs. 1-4.—Trypanosomes from 3 day liquid cow blood cultures. Figs. 5-9.—Specimens from 8 day liquid cow blood cultures. Fig. 10.—Specimen from 8 day liquid horse blood culture.

PLATE 15.

Figs. 11–20.—Trypanosomes from 14 day liquid cow blood cultures. Figs. 21–23.—Specimens from 20 day liquid cow blood cultures.

PLATE 16.

Figs. 24–35.—Trypanosomes from 4 day N. N. N. cultures.

PLATE 17.

Figs. 36–41.—Trypanosomes from 9 day N. N. N. cultures.

(Glaser: *Trypano on a amer canum.*)

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(Glaser: *Trypanosoma americanum.*)

(Glaser. *Trypanosoma americanum*.)

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(C a cr: *Tr\pan\\oma americanum.*)
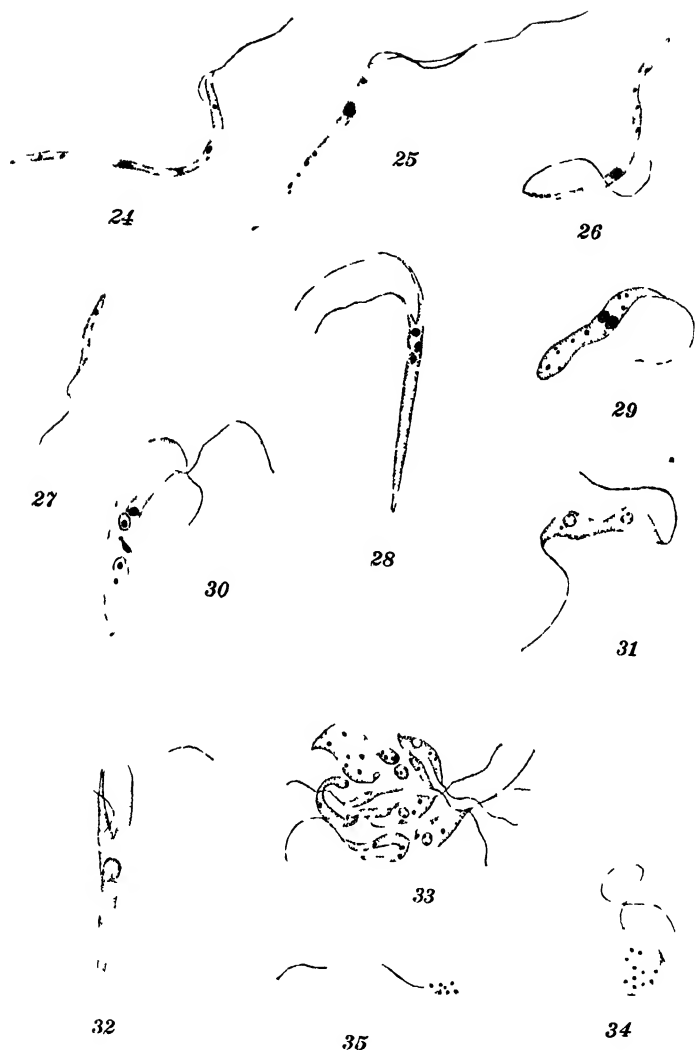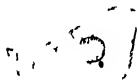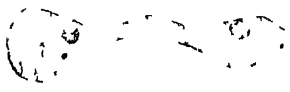
HEMOLYTIC ACTION OF A STAPHYLOCOCCUS DUE TO A FAT-SPLITTING ENZYME.

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In the routine examination of a sample of milk two dilutions plated in blood agar showed different effects. The plate of a 1:100 dilution indicated a pure culture of a non-hemolytic staphylococcus, while the plate of a 1:10 dilution appeared to be a pure culture of a hemolytic staphylococcus In the latter case the deep as well as the surface colonies showed clear hemolytic zones with a few corpuscles remaining next to the colony. This hemolysis is unusual for a staphylococcus which, ordinarily, even when hemolytic on the surface does not give evidence of hemolysis around the deep colonies. A culture was prepared by inoculation from a single deep hemolytic colony. The platings were repeated with this culture, and the same results obtained. These observations indicated that some constituent of the medium was responsible and not the presence of both a hemolytic and a non-hemolytic organism. The following study was, therefore, made to determine more accurately the precise factors involved.

The relation of the milk to the hemolytic process was outlined by plating the culture on (*a*) plain blood agar, (*b*) blood agar plus 0.1 cc. of sterile fat-free milk, (*c*) blood agar plus 0.1 cc. of whole milk, and (*d*) blood agar plus 0.02 cc. of cream. The results given in Table I show that hemolysis did not occur around the deep colonies in either the plain blood agar or in that to which fat-free milk had been added, but it did occur in the plates made with whole milk and with cream. Similar results occurred when bouillon was used instead of agar. They indicated that the effect of the dilution noted originally corresponded to a lowered fat content. The problem, therefore, resolved itself into a question of the relation of the fat to hemolysis and the action of the organism on the fat.

Characteristics of the Organism from Milk, Designated Staphylococcus A.—The organism is a small Gram-positive coccus, occurring often in pairs and irregular masses, and frequently in groups of three. On an agar slant the growth is smooth, opaque, and slightly yellowish white. On a blood agar plate the surface colonies are round, opaque, smooth, and more distinctly yellowish in color. In plain bouillon there is a good clouding, the growth adheres to the sides of the tube often forming a ring at the surface, and there is a small amount of sediment. Gelatin is liquefied after about 2 weeks. Milk is coagulated in 5 to 15 days. Acid is formed in glucose, lactose, sucrose, maltose, and mannitol bouillon, but no gas is produced. All these characteristics identify the organism as a staphylococcus.

TABLE I.

Hemolytic Action of Staphylococci in Media Containing Defibrinated Horse Blood with and without Cream.

	Control without milk.	Fat-free milk. 0.01 cc.	Whole milk 0.01 cc.	Cream 0.002 cc.
Blood agar plus culture (plated)........	−*	−	+	+
" bouillon plus culture.............	−	−		+++
" "	−	−		−

In the case of the agar the results are given for the deep colonies only. The quantities of milk, etc., indicated are those added to 1 cc. of media.

* In all the tables + indicates hemolysis; − no hemolysis.

Preparation of Blood.—The blood corpuscles obtained from horse blood were washed with 0.85 per cent salt solution and suspended in this fluid made up to the original volume of the defibrinated blood. It was added in the proportion of 1 drop to a cc. of bouillon. Whole defibrinated horse blood was used in the agar plates, about 1 cc. to each plate.

Standard agar was used for the plates in 10 to 12 cc. amounts. Standard bouillon was used for the bouillon cultures. 1 cc. of bouillon was measured into each tube. 0.02 cc. of 15 per cent cream diluted to 1:10 and 1 drop of blood were added to each cc. All tubes were incubated at 37°C. over night.

The results of the experiments are contained in Tables I to VI, The nature of the hemolytic zone on the blood agar plates suggested that the active hemolytic agent diffused out from the colonies; *i.e.*, that the process was extracellular since the hemolytic zone extended for a considerable distance beyond the colony. To determine the presence of an extracellular hemolytic agent the experiments summarized in Table II were carried out.

The staphylococci were killed and any extracellular enzymes present left unharmed. Such a procedure excluded the use of heat. We adopted ether, and later chloroform for control work, as an agent which would destroy the staphylococcus and which could then be removed from the media and thus would not interfere with the subsequent experiments because of its own hemolytic action. The

TABLE II.

Hemolytic Action of Bouillon Cultures of Staphylococci Sterilized with Ether or Chloroform to Which Horse Blood Corpuscles and Milk Fat Were Added.

	Cream (15 per cent) 0.002 cc.	Fat-free milk 0.01 cc.	Control without fat.
Etherized bouillon culture plus corpuscles..........	+++	−	−
Chloroform " ✓ " " "	+++	−	−

preparation of these etherized cultures was as follows: The organism was grown in standard bouillon 24 to 48 hours. Ether was added and the culture fluid shaken and allowed to stand at room temperature. The next day the sterility was tested by inoculating a tube of bouillon with 2 or 3 drops of the etherized culture. The ether was driven off by blowing air into the tube through a sterile pipette plugged with cotton. This procedure was repeated three or four times by alternately warming the culture in the incubator and then blowing air through it while cooling. This drove out practically all ether. That the specific effect of the ether was eliminated was indicated by the control tests. The results obtained with the etherized cultures, and confirmed with cultures treated with chloroform, show that after the staphylococcus has grown in bouillon the agent destroying the living organism does not destroy the substance which, in conjunction with

the cream or fat, produces hemolysis of red blood corpuscles. In the absence of cream or a fat hemolysis fails to occur.

It seemed evident that we were dealing with a lipolytic enzyme. In such a case if sufficient heat be applied either to the living culture or to the killed culture hemolysis should be prevented. The data indicating the effect of heat are summarized in Table III.

TABLE III.

Hemolytic Action of Both Living and Killed Bouillon Cultures of the Staphylococcus Which Had Been Heated before the Cream and Blood Corpuscles Were Added.

	Temperature.	Time	Hemolytic action.
	°C.	min.	
Living culture...........................	Unheated.		+++
	45	30	++*
	65	30	—
	100	5	—
Culture killed by ether....................	Unheated.		++
	45	30	++
	55	30	++
	65	30	—
	100	5	—
" " " chloroform................	Unheated.		+++
	45	30	+++
	55	30	+++
	65	30	—
	100	5	—

Final concentration of cream was 0.002 cc. per cc. of culture fluid.
*After heating to 45°C. the culture continued to grow.

It appeared at first that hemolytic action in the etherized culture was destroyed when the fluids were heated to 45°C. Later tests showed that the first results were due to the fact that the fat added to the tube after heating rose to the surface and was, therefore, acted upon very slowly. By occasionally shaking the tubes hemolysis occurred in tubes heated at 45°C. and also at 55°C., but at 65°C. hemolytic action was destroyed.

Having found that the ultimate hemolytic process was extracellular and thermolabile the next step was to eliminate the enzyme from the

final stage of hemolysis. This was done by allowing the living staphylococcus or the etherized culture to act upon cream or fat and then to destroy the organism or enzyme with heat before adding blood corpuscles. It was found that when the living culture or the etherized culture fluid was heated to 100°C. for 5 minutes neither of the fluids thus treated would hemolyze red blood corpuscles. On the other hand, when the living culture was in contact with cream for 4 hours and the etherized culture with cream for 18 hours and these fluids were then heated to 100°C. for 5 minutes hemolysis resulted. Success with this phase of the problem was no doubt due to the absence of complex protein material in the bouillon. To demonstrate this fact small amounts of serum were added to previously heated tubes of bouillon which were hemolytically active, and the resulting mixture was heated to 100°C. The addition of 1 or 2 drops of serum to 1 cc. of bouillon had no effect upon the hemolytic action of the bouillon; the addition of 4 drops resulted in a slight hemolysis, while the addition of 7 drops of serum resulted in a failure of the heated culture fluid to hemolyze the red blood corpuscles.

The substrate for the action of the lipase in the previous experiments was cream. To eliminate cream as a factor *per se*, other fats were tested with the staphylococcus and with the etherized culture fluid, such as butter, olive oil, nut butter, triolein, triacetin, tributyrin, and pork fat. The results are contained in Table IV.

Preparation of Fats.—Preliminary experiments suggested that a high degree of emulsification of fat was desirable. Soaps of certain fatty acids will lake red blood cells, consequently emulsification with an alkali was not feasible. Fair results were obtained by shaking various fats or oils with bouillon. The quantity of fat used was such that the concentration would approximate the quantity of fat added to the culture when 15 per cent cream was used.

Cream was drawn from the top of a tube of milk after it had stood several hours. This was sterilized fractionally in an Arnold sterilizer at 100°C. for 20 minutes on 3 successive days. For use it was diluted to 1:10 with sterile bouillon, making approximately a 1.5 per cent suspension. Fat-free milk was obtained by centrifuging and lifting off the cream with a spatula. This was sterilized fractionally. A suspension of olive oil was made by adding 1.5 cc. to 10 cc. of bouillon

and shaking in a mechanical shaker 1 to 2 hours. Suspensions of other fats, such as butter, nut butter, triacetin, triolein, tributyrin, and pork fat, were made in a similar way in bouillon and shaken. The final concentration of the fats in the experimental tubes was approximately 0.002 cc. per cc. of bouillon.

Positive results were obtained with emulsions of butter, olive oil, and triolein. With some fats the hemolytic action was less marked than with others. The difference in the action seems to be due, in part at least, to the character of the emulsion, for with those fats which were associated with hemolysis the more highly emulsified they were the better the results; furthermore, a shaken tube would often give

TABLE IV.

Hemolytic Action of Living and Etherized Bouillon Cultures of the Staphylococcus to Which Were Added Horse Blood Corpuscles and Various Fats.

	Cream.	Butter.	Olive oil.	Tributyrin.	Triolein.	Triacetin.	Nut butter.	Pork fat.	Fat-free milk.	Control without fat.
Living bouillon culture plus corpuscles............	+++	+	++	−	++	−	−	−	−	−
Etherized bouillon culture plus corpuscles.........	++	+	+		+		−	−	−	−

Final concentration of fat was approximately 0.002 cc. per cc. of bouillon.

a greater degree of hemolysis than an unshaken tube. There is considerable evidence in the literature to the effect that the salts of unsaturated fatty acids hemolyze red blood corpuscles more readily than the saturated fatty acids. Our results are, in general, in harmony with such findings.

To confirm our results with Staphylococcus A other strains indicated below were tested.

Staphylococcus B: A strain recently isolated from the milk of a cow. This cow and the one from which Staphylococcus A was isolated were in the same herd. The characteristics are in general the same as those for Staphylococcus A.

Staphylococcus C: An organism from our collection of stock cultures, isolated from a lung abscess of a calf in 1918. It liquefied

gelatin but not blood serum, and showed the yellowish color of a *Staphylococcus aureus.*

Staphylococcus D: A non-hemolytic organism also from our collection, isolated from purulent milk in 1899. It shows a yellowish pigment characteristic of a *Staphylococcus aureus.*

The experiments previously described were repeated with the different strains. The data for the bouillon culture experiments are contained in Table V.

TABLE V.

Hemolytic Action of Different Strains of Staphylococci in Bouillon Containing Defibrinated Horse Blood with and without Fat.

	Strain.	Incubation period.	Cream.	Butter.	Olive oil.	Tributyrin.	Triolein.	Triacetin.	Fat-free milk.	Control without fat.
		hrs.								
	Staphylococcus B	24	++++	+	+++	−	+++	−	−	−
		48	++++	+++	++++	−	++++	−	−	−
Blood bouillon plus culture..	" C	24	++	+	±	−	+	−	−	−
		48	++++	++	+	−	++	−	−	−
	" D	48	−	−	−	−	−	−	−	−
Blood bouillon only.			−	−	−	−	−	−	−	−

Final concentration of fat was approximately 0.002 cc. per cc. of bouillon.

The hemolytic action of Strains B and C was practically identical with that given for Staphylococcus A. Hemolysis occurred to a slight extent on the surface of agar plates in the absence of added fat, and was absent around the deep colonies or at most only a faint trace of hemolysis occurred. In the presence of cream, hemolysis was more extensive on the surface and occurred around the deep colonies. In bouillon, hemolysis occurred only in the presence of fat. With bouillon cultures killed with ether or chloroform the resulting fluid was able to effect hemolysis in the presence of cream. The fluids from cultures which had acted upon cream and had then been heated to 100°C.

were capable of hemolyzing red blood corpuscles. The active agent was destroyed by heating to 65°C.

Strain C which has been under cultivation for 3 years was not as active as the more recently isolated Strains A and B. The quantity of lipase elaborated in a given time was not so great, as evidenced by its slower rate of hemolysis in the bouillon culture tubes. In the tests on different kinds of fats it was found that hemolysis occurred with olive oil only after 65 hours.

TABLE VI.

Hemolytic Action of Staphylococcus A in Media Containing Blood Corpuscles from Various Animals with and without Cream.

		Horse.	Rabbit.		Sheep.	Cow.	Calf.
			A.	B.			
Agar plus culture	Without cream.	−	−	±	−	−	−
	With "	+	+	+	+	+	+
Living bouillon culture	Without "	−	+	+	+	+	−
	With "	+	+	+	+	+	+
Etherized bouillon culture	Without "	−		+	−		−
	With "	+		+	+		+
Heated bouillon culture (100°C.)	Without "						
	With "	−		+	−		−
Control with sterile bouillon	−		−		−	−	−

Final concentration of cream was approximately 0.002 cc. per cc. of bouillon. Results with agar are given for action around deep colonies.

Strain D was non-hemolytic and failed to produce hemolysis under any conditions.

The preceding experiments had been conducted with the corpuscles of one species of animal, the horse. Other animals were bled, such as sheep, rabbit, cow, and calf, and the experiments repeated with their corpuscles. The data are contained in Table VI.

In the presence of the blood corpuscles of sheep, rabbit, cow, and calf when the organism was plated on agar without the addition of cream, only a slight hemolysis occurred around the surface colonies

and no hemolysis around the deep colonies, except in the case of rabbit blood in which a very narrow hemolytic zone was present around the deep colonies. In those plates to which cream had been added hemolysis was more extensive around the surface colonies and took place around the deep colonies in a manner very similar to that for horse corpuscles. In plain bouillon with the corpuscles of sheep, rabbit, and cow, hemolysis occurred with the living culture of Staphylococcus A in the absence of added fat, while the result with calf corpuscles was like that with the horse. When the etherized culture fluid without cream was used, hemolysis did not occur with any of the blood corpuscles except in the case of those of the rabbit; in the presence of cream, hemolysis occurred in all cases and the extent of hemolysis with the rabbit corpuscles was much greater than in the absence of cream. Rabbit corpuscles were also hemolyzed by the heated culture. In view of the greater sensitiveness of the rabbit corpuscles, and the increased hemolysis in the presence of cream or added fat, it is not illogical to assume that there was sufficient fat in the bouillon to enable the staphylococcus to effect hemolysis through the hydrolysis of this fat. The difference between the blood corpuscles of various kinds may be due to the amount or character of fat contained in the corpuscles.

In the above experiment with the corpuscles of animals other than the horse it was noted that in the absence of cream slight hemolysis occurred around the surface colonies but not around the deep colonies. In the presence of cream, hemolysis occurred around the deep as well as the surface colonies. To eliminate the possible effect of oxygen of the air as a factor in the latter process agar shake cultures were made and pipetted into flattened test-tubes filled with the agar to a height of about 4 inches. Hemolysis took place in the bottom of such tubes as well as near the surface.

<center>DISCUSSION.</center>

From the experiments presented hemolysis in the case of the organism studied is shown to be associated with the presence of fat in the media. Cultures grown in plain horse blood bouillon or in the presence of fat-free milk are not hemolytic, while the cultures grown in the presence of cream or other fats are hemolytic.

The nature of the processes associated with the hemolytic action is brought out in the experiments dealing with culture fluids in which the organisms have been treated with ether, and by the effect of heat upon such fluids and on living cultures. Through the action of ether or chloroform, the organism itself is killed but this does not destroy the ability of the culture fluid to effect the hemolysis of the red blood corpuscles. If the living culture or an etherized culture fluid be heated neither of the resulting fluids is capable of hemolyzing the red blood corpuscles.[1] When a living culture or an etherized culture fluid of the staphylococcus is permitted to stand with cream or other fat for several hours and is then heated to 100°C. the resulting fluid is capable of producing hemolysis. We feel justified, therefore, in attributing the hemolytic effects to the direct action of fatty acids (or soaps) which have been formed from the cream or fat by an extracellular enzyme elaborated by the staphylococcus. The enzyme is not dialyzable, for hemolytic action could not be obtained with either living or killed cultures in collodion bags.

The occurrence of hemolysis due to the action of a lipase upon fat necessitates the consideration of two factors in the study of organisms, (a) the presence of a lipase and (b) the elimination of its lipolytic action before concluding that hemolysis is due to another type of hemolytic agent.

Many points brought out by our experiments have been observed separately by others but so far as we can find the hemolytic action of a staphylococcus has not been directly connected with its lipolytic activities. Several organisms were reported by Eijkman (1) in 1901 as producing a lipase and among these was given *Staphylococcus pyogenes aureus*. An association between hemolysis and lipolysis was demonstrated by Noguchi (2) in 1907 who showed that hemolysis may be a direct result of fat-splitting. He used various tissue lipases with a considerable number of fats, but the most satisfactory results were obtained with triolein, butter, and fatty mixtures which he extracted from the fat tissues and mesentery of dogs and guinea pigs. According to his report neither the fat nor the lipase alone produced hemolysis.

Von Liebermann (3) extracted a thermostable hemolytic substance from hog corpuscles which was found to be an acid or a mixture of acids. He did not identify the acids but was able to demonstrate similar hemolytic action with

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[1] There was one exception to this statement and that was the corpuscles of the rabbit (see page 587).

oleic acid. Heating inactivated oleic acid-serum mixtures. This agrees with the work of Noguchi who found that when his alcohol extracts (soap) were heated alone they were not inactivated but when heated with serum they were inactivated. Von Liebermann was able to reactivate such mixtures by the careful addition of acid.

Neuberg and Reicher (4) demonstrated the lipolytic action of various plant and animal substances by measuring the increase in acidity of mixtures of an hemolysin and a fat, particularly olive oil. These authors suggest that the hemolytic effects observed by von Liebermann may have been due to the fatty acid produced by the action of lipase upon the fats of red blood corpuscles. In our experiments the hemolytic effect is apparently not directly upon the red blood corpuscles, since in our control experiments hemolysis failed to occur in the presence of lipases but in the absence of added fat.

Noguchi (5) also extracted a hemolytic agent from animal tissues with alcohol which consisted of soaps. Fatty acids or their soaps are known to produce hemolysis. The smallest amount of fatty acid necessary for complete hemolysis of 0.5 cc. of a 5 per cent suspension of red blood cells was studied by McPhedran (6) in 1913. In our experiments we made no quantitative determinations but we used about 0.3 mg. of the fat, and probably this was not all split into fatty acids. McPhedran found 0.03 mg. of oleic, linoleic, dibromostearic, or the two isomeric monobromostearic acids sufficient for complete hemolysis of 0.5 cc. of a 5 per cent suspension of red blood corpuscles while palmitic acid and dihydroxystearic acids were required to be present in quantities ten times as great as for the above acids.

The hemolytic character of the staphylococcus is described in various text-books as due to a specific hemolysin, staphylolysin, but no mention is made of a fat-splitting enzyme.

SUMMARY.

A staphylococcus was isolated from milk which is hemolytic with horse blood only in the presence of fat. Similar results were obtained with two other strains of staphylococci.

The hemolysis is the result of the action of a fatty acid (or soap) upon the red blood cells. The fatty acid is formed by the action of a lipase elaborated by the staphylococcus.

The corpuscles of different animals show slight variations in the ease with which they are hemolyzed by the staphylococcus.

Attention is called to the desirability of testing for lipases in the study of staphylococci or of hemolysis.

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MODIFICATION OF AN IMPROVED ANAEROBE JAR.

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The explosion of one of our anaerobe jars after use for a year without accident has led to a slight modification of the apparatus previously described.[1] Reference to Text-figs. 1 and 2 of the article referred to will show the copper wire terminals B_1 and B_2 leading through the rubber stoppers C_1 and C_2 beside the glass tubing D. It has been found that after some months of use the copper wires become corroded where they are in contact with the rubber stoppers, probably due to the interaction of moisture, heat, and some constituent of the rubber stoppers (possibly sulfur). The accident referred to was due to the breaking of one of these corroded wires resulting in the production of a spark just outside the rubber stopper when the electric current was turned on. The corrosion may be avoided by having the copper wires enter the coil through the bore of the glass tube D, joining the nichrome wire A through small holes in the side of the tube within the coil. Although the capillary rubber tubing E_1 and E_2 used for insulation has been found to have no appreciable corrosive action on the copper wires it is further suggested that for B_1 and B_2 one may
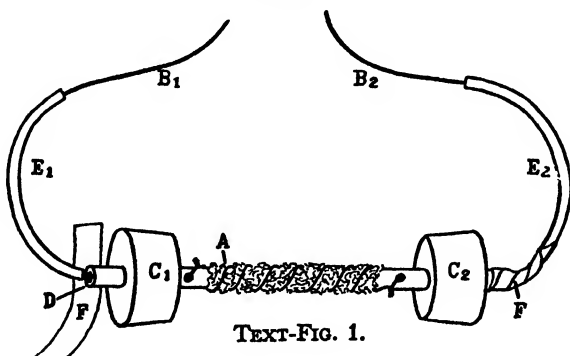
TEXT-FIG. 1.

use wire insulated with a non-corrosive insulation such as asbestos, thus eliminating the rubber tubing altogether. We also pack·the bore of the glass tube with asbestos at either end where the wires pass into it and wrap the joint with insulating tape (F). The modifications recommended are shown in Text-fig. 1.

[1] Brown, J. H., An improved anaerobe jar, *J. Exp. Med.*, 1921, xxxiii, 677.

THE VASELINE TUBE AND SYRINGE METHOD OF MICRO GAS ANALYSIS OF BACTERIAL CULTURES.

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Plates 55 to 57.

The methods to be described have been evolved not so much because of dissatisfaction with results which could be obtained by methods previously used but rather for reasons of greater convenience and economy. It is for these reasons that the Smith fermentation tube has continued in use long after its limitations have been recognized. The Smith tube was never recommended as a means for accurate study of the gas-forming function of bacteria. It was recommended as an instrument for collecting and approximately measuring and analyzing the gases which are given off by certain cultures when grown in this tube. The result of such measurement and analysis was found to be a means of distinguishing certain broad groups of bacteria. The gas was produced under the conditions imposed by the tube and the gas formula $\left(\dfrac{H_2}{CO_2}\right)$ was understood to be valid for those conditions only. Critics of the Smith fermentation tube should not lose sight of these facts.

More efficient and more elaborate forms of apparatus have been devised by various authors with a different purpose in view, namely to collect all the gas which a culture forms, to measure both that given off from the medium and that held by the medium, and to make very accurate analyses of these gases. I refer particularly to the apparatus described by Keyes (1909), Frieber (1913, *b*), Rogers, Clark and Davis (1914), and Wolf and Harris (1917). These methods are much better adapted for the careful study of the gas-forming activity of a

few cultures than for the comparative study of a large number of strains. Doubtless the apparatus described by Van Slyke and Stadie (1921) could be used for bacterial cultures and should yield more accurate results than the syringe. However, it is believed that the method to be described retains the simplicity of the Smith tube, at least something of the greater accuracy of the more elaborate methods, and some advantages for the bacteriologist possessed by none of the older methods.

During a study of anaerobic bacteria still in progress, use was made of small amounts of media covered by vaseline in test-tubes. It was noticed that as gas was formed by a culture the vaseline was forced up the tube, the vaseline plug remaining perfectly intact and acting as a self-lubricating piston at the temperature of the room or incubator. We have continued to use this form of culture tube in the manner to be described.

Measurement of Gas above the Medium.

A record of the amount of gas present above the medium may be made at any time by marking with a wax pencil on the side of the tube the lower level of the vaseline plug. To measure the amount of gas in the tube a device somewhat like the Frost gasometer for the Smith fermentation tube is used. The gasometer shown in Text-fig. 1 is made of sheet tin or brass. The lower edge is turned at a right angle towards the observer so that a shelf is formed on which rests the bottom of the tube being examined. The base-line of the graduations ruled on the surface of the gasometer is shown as a double line marked 0.0 and placed diagonally with reference to the shelf and above it. The culture tube is pushed along the shelf until the meniscus of the culture medium rests at the base-line. The graduations above the base-line radiate from a point on the base-line at some distance to the right of the figure so that when the tube cuts the base-line vertically the amount of gas between the medium and the vaseline plug can be read as volumes and tenths of a volume of the amount of medium present in the tube, or, if multiplied by 100, as volumes per cent. If the culture produces sufficient gas to force the vaseline up nearly to the cotton plug the amount of gas is recorded, then by directing the flame of a micro burner against the side of the tube the vaseline is

melted and allowed to drop down onto the surface of the medium and to solidify again. This may be done repeatedly in which case the total gas formed above the medium is obtained by addition of the measurements recorded before each melting of the vaseline plug.
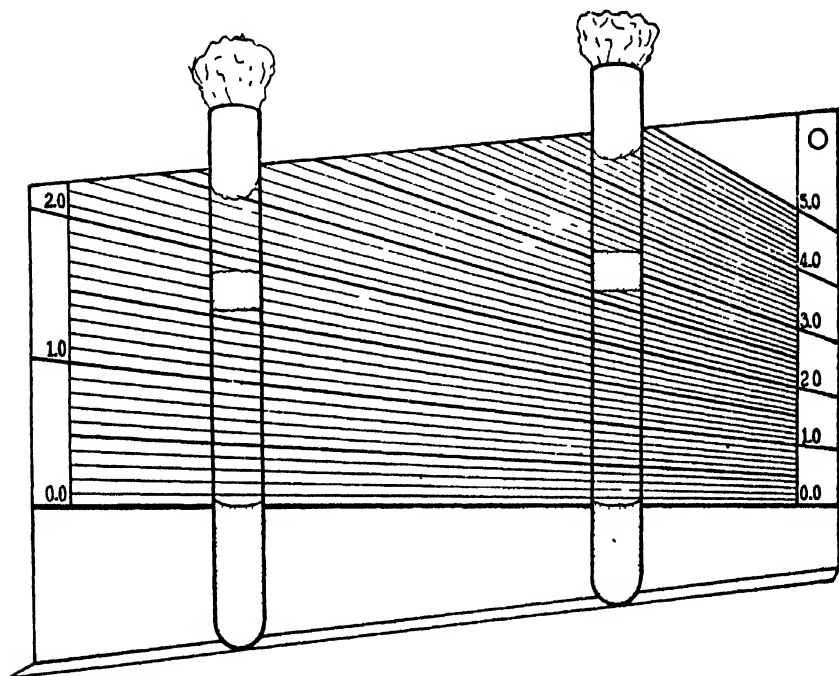
TEXT-FIG. 1. The gasometer with two tubes in position for the measurement of the gas above the medium in terms of the volume of medium employed.

Determination of CO₂ in the Gas above the Medium.

By means of a Luer tuberculin syringe with a long fine needle[1] attached by means of a short length of capillary rubber tubing a sample of gas may be withdrawn and the CO_2 determined in the syringe. The technique and necessary equipment are illustrated in Figs. 1 and 2. The various steps of the technique are as follows:

[1] The needle used was Gauge 20 and 6 inches long, specially made for us by Becton, Dickinson and Co., Rutherford, N. J. There is also needed a small adapter such as that listed by Arthur H. Thomas Co., No. 9419, for 606 Hose End to Luer Slip Needles.

1. Rinse the syringe and needle with dilute acid which is then expelled as completely as possible by forcing air into and out of the syringe a number of times. Push the syringe plunger in as far as it will go or, better, to an exact reading at the lower end of the scale on the barrel of the syringe.

2. Dry and sterilize the needle by passing it through the flame a number of times.

3. With the usual precautions to maintain sterility draw the cotton plug from the culture tube and replace it with the needle held between the cotton plug and the side of the tube.

4. Push the needle down until the point passes through the vaseline plug and well into the zone of gas.

5. With the syringe held as shown in Fig. 1 slowly withdraw a measured quantity of gas (something less than 1 cc. so that when step 7 is taken the total gas can be read between the plunger and the meniscus). The vaseline plug will be seen to move down the tube as the gas is withdrawn.

6. Leave the cotton plug in place and pull the needle up until the point remains in the upper part of the vaseline. With a small hot spatula touch the outside of the test-tube opposite the track of the needle in the lower part of the vaseline plug. Leave the spatula in contact with the tube just long enough to close the opening in the vaseline.

7. Withdraw the needle from the tube, immediately stick the end into the dilute acid, and with the syringe held vertically draw up acid until the meniscus can be seen and read against the scale on the lower end of the syringe. Read the exact amount of gas (and air) in the syringe between the plunger and the meniscus of acid.

8. Stick the end of the needle into a 2 or 3 per cent solution of sodium hydroxide. With the apparatus in the position shown in Fig. 2 draw out the plunger until the alkali begins to enter the barrel of the syringe and neutralizes the acid there. As carbon dioxide is absorbed more alkali is automatically drawn into the syringe without further use of the plunger. With the end of the needle still in the alkali solution rock the syringe back and forth a number of times to insure complete absorption of the CO_2.

9. Again hold the syringe vertically with the plunger uppermost and read the amount of residual gas in the syringe. The difference between this reading and the one taken in step 7 equals the amount of CO_2 in the sample taken. The percentage of CO_2 in the sample is easily calculated and may be expressed as a gas ratio $\left(\dfrac{H_2}{CO_2}\right)$ if so desired.

We have tried a number of slight modifications of the details of the above technique but the above is probably the most perfect. The accuracy of the method is apparently limited only by the fineness of the graduations on the syringe. These should be at most 0.01 cc.

We are able to get almost as accurate results with 0.1 cc. of gas sample as with 1.0 cc. It is possible to determine the CO_2 content of a good sized bubble beneath the vaseline plug or in an agar shake culture. It is an advantage to select a tuberculin syringe having the following characteristics: (a) finely cut graduations, (b) a long slender barrel with considerable space above the graduations, (c) a well fitting plunger of colored glass with a sharp square-cut end which can be accurately read against the graduations on the colorless glass barrel. The carbon dioxide content of the small amount of atmospheric air contained in the needle and capillary rubber tube is a negligible quantity well within the limits of error. If the cultures are allowed to cool down to room temperature before the gas analysis is made and if the acid and alkali solutions are kept at the same temperature the temperature factor is also negligible. Formerly we took the precaution of allowing the end of the needle to be sealed by the vaseline (melted by the hot spatula) as it was withdrawn from the tube and then pricking this seal just as the needle was placed into the acid but it has been found unnecessary. There is no appreciable interchange of air and gas through the needle during the short time it is exposed to the air while being transferred from the culture tube to the dilute acid.

Determination of Carbonates and CO_2 in the Medium.

The carbonates and CO_2 in a fluid medium may be determined by means of the same apparatus. The equipment and technique are illustrated in Fig. 3. The steps of the technique are as follows:

1. Rinse the syringe and needle thoroughly with distilled water.
2. Dry and sterilize the needle in the flame.
3. With aseptic precautions place the needle so that it is held between the cotton plug and the side of the tube as for the determination of CO_2 above the medium.
4. Having placed the end of the syringe plunger at one of the graduation marks on the lower end of the barrel push the needle down the side of the tube through the vaseline plug (if vaseline is used) until the point is in the medium.
5. Draw the plunger of the syringe back just 0.1 cc.
6. Withdraw the needle with the same precautions as described in step 6 for the determination of CO_2 above the medium.
7. Draw into the needle about 0.03 cc. of air.

8. Stick the point of the needle into a small vial of capryl alcohol (colored with scarlet R) and draw 0.01 cc. into the needle, followed by 0.01 cc. of air.

9. Draw into the needle 0.05 cc. of 5 per cent sulfuric acid (colored with methyl red).

10. Holding the syringe vertically with the open needle uppermost draw back the plunger just until all of the red capryl alcohol and sulfuric acid are seen to be within the barrel of the syringe.

11. With the index finger of the left hand tightly stop up the end of the syringe where the rubber tube is attached and holding it as shown in Fig. 3 draw down the plunger while with a partial vacuum within the syringe the air leaks by the plunger and passes up through the contents of the syringe in a stream of fine bubbles. When the end of the plunger reaches a position a little beyond the graduations it should be allowed to remain here until the air ceases bubbling.

12. Turn the syringe to a vertical position with the plunger uppermost, release the finger from the rubber tube, push in the plunger to the beginning of the grad-uation marks, wait a few seconds to allow the fluid to drain down the sides of the syringe, and read the amount of gas and air present between the meniscus and the plunger.

13. Without changing the position of the syringe, needle hanging down, expel most of the fluid until the meniscus reaches the bottom of the syringe. Without admitting any air dip the end of the needle, now filled with fluid, into the sodium hydroxide solution and with the syringe held in the position shown in Fig. 2 draw up some of the alkali. Rock the syringe back and forth a few times. Again expel most of the fluid. holding the syringe in a vertical position and taking care to expel none of the gas. Draw in a fresh portion of sodium hydroxide and rock again.

14. Finally, holding the syringe vertically with needle hanging downward push the plunger in until its end reaches the graduation marks and read the amount of air remaining in the syringe. The difference between this reading and the one taken in step 12 equals the amount of CO_2 extracted from 0.1 cc. of medium, best expressed as volumes or as volumes per cent; e.g., 0.1 cc. of CO_2 from 0.1 cc. of medium equals 1 volume or 100 volumes per cent.

It is our experience that more accurate results are obtained with samples of 0.1 cc. of medium than with larger amounts. This is doubtless due to the fact that in such a small syringe as we use the amount of air that can be passed through the sample and held within the syringe for measurement is limited and although sufficient for complete aeration of 0.1 cc. of medium is hardly sufficient for a larger sample. Without doubt larger samples could be employed in a longer syringe. It is an advantage to color the acid and alkali solutions with indicators, methyl red in the acid and thymol blue in the alkali, so that one may be sure of the reaction of the contents of the syringe at all times.

Accuracy of the Determinations.

The determinations in Table I were made from a single culture of an anaerobic organism in 5 cc. of plain bouillon under vaseline. The results illustrate the possibility of obtaining uniform results with multiple determinations and various methods of expressing the results.

TABLE I.

Multiple Determinations of Carbon Dioxide Produced by an Anaerobic Organism in Plain Bouillon.

Initial CO_2 content of medium = 0.0

Final hydrogen ion concentration of culture = pH 6.5

Total gas above medium at 37°C. = 1.05 volumes per 1.0 volume of medium.

Determinations of CO_2 above medium.						
Sample, *cc.*	0.75	0.75	0.45	0.45	0.1	0.1
CO_2, *per cent.*	41.0	40.0	40.0	39.0	40.0	39.0
$\dfrac{H_2}{CO_2}$	$\dfrac{59}{41}$	$\dfrac{60}{40}$	$\dfrac{60}{40}$	$\dfrac{61}{39}$	$\dfrac{60}{40}$	$\dfrac{61}{39}$
CO_2 (vol. at 37°C.)	0.43	0.42	0.42	0.41	0.42	0.41

Determinations of CO_2 in medium* (samples = 0.1 cc.).						
CO_2 (vol. at 20°C.)	0.55	0.5	0.5	0.55	0.5	0.5

Computation of total CO_2.

Average; CO_2 above medium .... 0.42 vol. at 37°C.

" CO_2 in medium .... 0.52 vol. at 20°C. or 0.55 " " 37° "

Total CO_2 per 1.0 vol. of medium .... 0.97 " " 37° "

* By CO_2 in medium is meant not only CO_2 present as such but also that present in the form of carbonates or carbonic acid.

To determine how much of the CO_2 present as carbonate might be recovered by the above method from water or from bouillon the following experiment was performed. 0.5066 gm. of sodium oxalate was converted into sodium carbonate by ignition in a platinum crucible. The sodium carbonate was dissolved in 10 cc. of distilled water and 3 cc. of this solution were diluted to 25 cc. with distilled water and with plain bouillon respectively for the determinations to be described.

As a control 3 cc. of distilled water were added to 22 cc. of the bouillon without addition of carbonate. Quadruple determinations were made of the CO_2 recovered from each of the three solutions. The amount of each sample taken was 0.1 cc. The results are presented in Table II.

TABLE II.

Determinations of Carbon Dioxide in Standard Solutions of Sodium Carbonate in Water and in Bouillon.

Room temperature = 20°C. Barometric pressure = 765.2 mm. Hg.

Hydrogen ion concentration of bouillon before addition of Na_2CO_3 = pH 7.3

	Determinations.				Average.
CO_2 from Na_2CO_3 in water.............	1.05	1.05	1.05	1.03	1.045 vol. in 1.0 vol. of solution.
CO_2 " Na_2CO_3 " bouillon...........	1.02	1.05	1.05	1.05	1.042 vol. in 1.0 vol. of solution.
CO_2 " H_2O " "	0.02	0.0	0.03	0.0	0.012 vol. in 1.0 vol. of solution.

Calculated theoretical volume of CO_2 in the above solutions of Na_2CO_3 in water and in bouillon = 1.066 volume in 1.0 volume of solution at 20°C. and 765.2 mm. Hg.

Experiments Illustrating the Technical Possibilities of the Methods.

Although the conditions under which a culture grows in the vaseline tube are not the same as those in the Smith fermentation tube and one would therefore not expect to obtain identical results in the two tubes, it seemed worth while to compare the results obtained by both methods with a view to interpreting their differences. A number of series of experiments have been performed with this end in view. The following is typical.

A strain of *Bacterium coli* was inoculated into six vaseline tubes and five fermentation tubes containing a certain lot of 2 per cent dextrose bouillon (pH = 7.2). By the 4th day of incubation all tubes had ceased to show changes in gas volume. The results of analysis of the gas above the media on the 4th day are recorded in Table III.

It should be noted that the total volume of gas produced above the medium in the two kinds of tubes cannot be compared because the

gas is measured in different terms. In the fermentation tube the gas collected comes from a diminishing amount of culture as gas formation forces the medium over into the open bulb and the gas is measured in terms of the capacity of the closed arm of the tube. In the vaseline tube the volume of the culture medium remains constant and the gas is measured in terms of volume of the medium. It is for this reason

TABLE III.

Gas Formation by Dextrose Bouillon Cultures of Bacterium coli in Smith Fermentation Tubes and in Vaseline Tubes.

Fermentation tubes.		Vaseline tubes.	
Gas above medium.	$\dfrac{H_2}{CO_2}$	Gas above medium.	$\dfrac{H_2}{CO_2}$
per cent		*vol.*	
65	$\dfrac{53}{47}$	0.95	$\dfrac{48}{52}$
41	$\dfrac{55}{45}$	0.9	$\dfrac{49}{51}$
46	$\dfrac{55}{45}$	0.9	$\dfrac{50}{50}$
49	$\dfrac{51}{49}$	0.9	$\dfrac{50}{50}$
51	$\dfrac{56}{44}$	0.9	$\dfrac{50}{50}$
		0.9	$\dfrac{50}{50}$

that we prefer to avoid the use of the term "per cent" in stating the volume of gas produced in the vaseline tube. It is difficult to see how one set of values can be translated into terms of the other. Aside from this fact it is noted that the results in the vaseline tubes are somewhat more uniform than those in the fermentation tubes. It is also found that invariably a larger proportion of CO_2 is formed under vaseline than in the closed arm of the fermentation tube. The reason for this is, as pointed out by Keyes (1909), that CO_2 is much more

soluble in water than is hydrogen and therefore not only does a considerable volume of CO_2 pass into solution in the medium but in the fermentation tube passes through the medium out into the air.

As a means of studying gas production the vaseline tube has many points of similarity to the long agar tube of Burri and Düggeli (1909) used by Frieber (1913, *a* and *b*) for gas analyses. In this tube, however, only solid medium was used and a layer of sterile non-nutrient agar was used instead of vaseline as a seal. The technical possibilities of the vaseline tube are much greater.

TABLE IV.

Gas Formation by Dextrose Bouillon Cultures of Bacterium coli in Vaseline Tubes under Anaerobic and Aerobic Conditions.

Vaseline tubes (without air).		Vaseline tubes (plus 1.0 vol. of air).	
Gas above medium.	CO₂	Gas above medium.	CO₂
vol.	*per cent*	*vol.*	*per cent*
0.95	52	1.95	38
0.9	51	1.9	38
0.9	50	1.95	36
0.9	50	1.9	38
0.9	50	1.95	35
0.9	50	1.95	36
Average.......0.91	50.5	1.93	36.8
0.91 × 0.505 = 0.46 vol. of CO₂		1.93 × 0.368 = 0.71 vol. of CO₂	

In the methods of all the authors referred to above the cultures were grown under anaerobic conditions. Eldredge and Rogers (1914) and Osterhout (1918) have devised forms of apparatus for studying CO_2 production under aerobic or at least partially aerobic conditions. Some of the results obtained by the use of the tube of Eldredge and Rogers will be referred to later. The vaseline tube also permits the study of gas production under controlled aerobic conditions. After the medium in the tube has been inoculated and the vaseline allowed to solidify on the surface a measured volume of air or other gas may be injected beneath the vaseline plug by means of a syringe and the long needle used for gas analysis. The passage made by the needle in the vaseline is easily closed by means of a warm spatula as the needle

is withdrawn. In the experiment partially recorded in Table III was also included a series of six similar vaseline tubes of the same medium inoculated with the same strain of *Bacterium coli* but injected with an equal volume of air just after inoculation. Gas analyses were made at the same time as were those recorded in Table III. A comparison of the results with those of the vaseline tubes without air is given in Table IV.

Experiments were carried out with various volumes of air injected under the vaseline plug. With decreasing amounts of air below 1 volume the results gradually approach those obtained under anaerobic conditions. With 2 volumes of air the results were very nearly the same as with 1 volume. Naturally these proportions must be regarded as valid for this culture and medium only.

If from the 1.93 volumes of gas found in the tubes containing air the volume of air injected be subtracted, there remains 0.93 volume of gas produced by the culture, and since 0.71 volumes of CO_2 were present it might be computed that the gas ratio under aerobic conditions was $\dfrac{H_2}{CO_2} = \dfrac{22}{71}$, or $\dfrac{24}{76}$, as compared with $\dfrac{H_2}{CO_2} = \dfrac{50}{50}$ under anaerobic conditions. This calculation, however, assumes that the air injected is entirely inert and takes no part in the reaction. This we have reason to believe is not the case.

Determination of Oxygen in the Gas above the Medium.

The oxygen is readily determined in the same sample of gas used for the determination of CO_2 provided no atmospheric air is drawn into the syringe. This may be accomplished by having the space between the syringe plunger and the end of the needle filled with dilute acid instead of air as the needle is thrust through the vaseline plug. Otherwise the CO_2 is determined in the same manner described above and then the needle is dipped into a concentrated aqueous solution of pyrogallic acid. A little of the pyrogallic acid solution is drawn up into the syringe and as it mixes with the sodium hydroxide and absorbs the oxygen present more of the pyrogallic acid is automatically sucked up. The process is slower than the absorption of CO_2 by sodium hydroxide but, with constant rocking of the syringe back and forth, is complete in 2 or 3 minutes and the reading is made.

In another experiment with the same strain of *Bacterium coli* as used for the results given in Tables III and IV, both the CO_2 and the O_2 above the medium were determined and also the CO_2 in the medium. The results are given in Table V.

The outstanding feature of this experiment was the disappearance of the oxygen from the air above the aerobic culture. Assuming that

TABLE V.

Further Gas Analysis of Dextrose Bouillon Cultures of Bacterium coli under Anaerobic and Aerobic Conditions.

	Vaseline tubes (without air).		Vaseline tubes (plus 1.15 vol. of air).	
	Culture.	Control.	Culture.	Control.
	vol.	*vol.*	*vol.*	*vol.*
Gas above medium............	1.1	0.0	2.15	1.15
CO_2 " "	0.5	0.0	0.75	0.04
CO_2 in "	0.25	0.05	0.15	0.05
Total CO_2....................	0.75	0.05	0.9	0.09
O_2 above medium............	0.0	0.0	0.0	0.07

Oxygen determined in atmosphere = 0.2 vol.

this was the case also in the experiment of Table IV, consideration of this factor would increase the gas ratio under aerobic conditions to $\frac{H_2}{CO_2} = \frac{37}{63}$ instead of $\frac{24}{76}$ since the space assumed to be occupied by oxygen was in reality occupied by some gas[2] other than CO_2 or O_2 produced by the culture. The disappearance of a part of the oxygen in the aerobic control tube noted in Table V was probably due to its passing into solution in the air-free medium which had been kept tightly sealed since it was autoclaved. The small amounts of CO_2 found in the controls may or may not be significant. It is not surprising that less CO_2 was found in the medium of the aerobic culture than in the anaerobic culture since the presence of air above the former favored the outward diffusion of CO_2. Taking all the figures into

[2] In stating the gas ratio $\left(\frac{H_2}{CO_2}\right)$ we have followed the custom of regarding all of the gas produced by the culture, other than CO_2, as hydrogen.

consideration it appears that *Bacterium coli* not only produces more CO_2 under aerobic conditions but a larger proportion of CO_2 with respect to H_2 or other gases.

Certain organisms which have been regarded as non-producers of gas have been found by special methods to produce appreciable amounts of CO_2. Such results are interesting and open up new possibilities for classification and for physiological study. However, they do not invalidate the practical value of formerly used methods for distinguishing between so called "gas-producing" and "non-gas-producing" organisms under stated conditions of cultivation. It was shown by Hesse (1893) that *Bacterium typhosus* and many other bacteria ordinarily considered to be non-gas producers do produce an appreciable amount of CO_2 and consume O_2 in the process. He calls this process the respiratory activity of bacteria.

Ayers, Rupp, and Mudge (1921) used the tube of Eldredge and Rogers (1914) to demonstrate CO_2 production by streptococci, and Nichols (1921) demonstrated CO_2 production by *Bacterium typhosus* by the same means. In this tube cultures are grown under aerobic conditions. Since, however, the atmosphere of the tube above the medium is kept free of CO_2 by the barium hydroxide solution we would expect to find less CO_2 in the medium than when the aerobic vaseline tube is used. To compare the results obtained in the vaseline tube with those of the Eldredge tube we must therefore consider the CO_2 in the medium as well as that above it in the vaseline tube. We have done this with two strains of streptococci for which we are indebted to Dr. Ayers. Strain X-4 was reported by Ayers, Rupp, and Mudge as producing CO_2 from dextrose and Strain 16H-1 was reported to produce CO_2 from Bacto-peptone. In Table VI are given the total CO_2 determinations of Ayers' strains of streptococci in various media. The determinations were made after incubation at 37°C. for 6 days. The amount of medium used was 3 cc. in each tube.

The resemblance between the CO_2 determinations of our aerobic cultures and those of Ayers, Rupp, and Mudge obtained by an entirely different method is so close that the differences seem insignificant. It is to be noted, however, that under anaerobic conditions much less CO_2 is produced in all media. Possibly this explains failure to demonstrate gas production by streptococci under the anaerobic conditions imposed by other methods.

Using the tube of Eldredge and Rogers, Nichols (1921) demonstrated CO_2 production by *Bacterium typhosus*. He obtained the maximum amount of CO_2 from cultures in 1 per cent glucose extract broth. In 2 per cent glucose veal infusion broth and with a different strain of typhoid bacillus, however, we have obtained even larger amounts of CO_2. The results of our determinations are recorded in Table VII.

TABLE VI.

Determination of Total Carbon Dioxide Produced by Streptococci in Vaseline Tubes under Anaerobic and Aerobic Conditions.

The medium was veal infusion bouillon containing 1 per cent Fairchild's peptone plus the substances indicated above each column.

Strain.	Vaseline tubes (without air).			Vaseline tubes (plus 1.0 vol. of air).		
	3 per cent Fairchild's peptone.	3 per cent Bacto-peptone.	2 per cent dextrose.	3 per cent Fairchild's peptone.	3 per cent Bacto-peptone.	2 per cent dextrose.
	vol.	*vol.*	*vol.*	*vol.*	*vol.*	*vol.*
Streptococcus X-4..........	0.05	0.05	0.67	0.13	0.06	1.17 (1.25)
" 16H-1........	0.05	0.13	0.0	0.21 (0.12)*	0.22 (0.33)	0.13 (0.09)

* The bold faced figures in parentheses are the results of Ayers, Rupp, and Mudge reduced to the same terms as ours. It should be noted, however, that whereas one of their media contained 4 per cent of Bacto-peptone ours used for comparison contained 3 per cent of Bacto-peptone and 1 per cent of Fairchild's peptone.

There was good growth of the typhoid bacillus under anaerobic conditions, though somewhat better under aerobic conditions. It is again seen that a really significant amount of CO_2 is produced under aerobic conditions only, and that a part but not all of the oxygen was consumed.

It must be pointed out that in interpreting the results of the determination of CO_2 in and above the medium certain very important factors must be taken into consideration. There is a very intimate relationship between temperature, hydrogen ion concentration of the culture, and the proportionate amount of CO_2 in and above the me-

dium. For instance, if the culture becomes alkaline, as may be the case with certain anaerobes, a large proportion of the CO_2 will be found in the medium as carbonates. If it becomes strongly acid a large proportion of the CO_2 will be found in the gas above the medium. If the culture is transferred from the incubator to the refrigerator the solubility of gases in the medium is appreciably increased. If the vaseline plug is broken so that atmospheric air gains access to the culture, CO_2 begins to pass out of the medium into the air. The latter can be demonstrated easily by breaking the seal of a culture and making periodic determinations of the CO_2 in the medium.

TABLE VII.

Determination of Carbon Dioxide Produced by Bacterium typhosus in Dextrose Bouillon under Anaerobic and Aerobic Conditions.

	Vaseline tube (without air).	Vaseline tubes (plus 1.0 vol. of air).	
	Culture.	Culture.	Control.
	vol.	*vol.*	*vol.*
Gas above medium..............	0.0	1.1	1.0
CO_2 " "	0.0	0.14	0.03
CO_2 in "	0.1	0.15	
Total CO_2.....................	0.1	0.29	
O_2 above medium...............	0.0	0.06	0.13

Oxygen determined in atmosphere = 0.2 vol.

Sterilization, Inoculation, and Cleaning of Vaseline Tubes.

Before summarizing the advantages of the technique described it may be well to describe one or two simple points of technique which serve to make the vaseline tube almost as convenient to handle as an ordinary test-tube.

Autoclaving cannot be relied upon to sterilize vaseline since it is essentially a "dry" substance which the steam does not penetrate. It may be perfectly sterilized without visible alteration along with glassware in the hot air sterilizer at 175–185°C. for 2 hours. After being so sterilized it may be pipetted onto non-sterile medium in the tubes and then autoclaved with the medium. During sterilization air is driven from the medium and if the tubes are promptly cooled

after coming from the autoclave the medium under the solid vaseline plug is preserved free from air for long periods of time. There is no danger of spilling if tubes are turned over, and the medium may be kept at room or incubator temperature without evaporation. Since there is no water vapor in the tubes above the vaseline there is no tendency for molds to grow through the cotton plugs.

It is not necessary to pass a pipette through the vaseline to inoculate or add anything to the medium in the tube. In fact it may be inoculated with a platinum loop if the vaseline seal is opened as shown in Figs. 4 and 5. The vaseline is melted by directing the flame of a micro burner against the side of the tube. The tube is then slanted in a dish or tray of water as shown in Fig. 4. After the vaseline has hardened as a layer over the slanted surface of the medium the tube is rotated and gently tapped by the fingers, as shown in Fig. 5, until the vaseline over the medium flaps up and adheres to the side of the tube, exposing the medium for inoculation. The seal is again closed by melting the vaseline by means of the flame directed against the outside of the tube in the region of the vaseline. The medium need not be perceptibly heated by the process and with reasonable care tubes rarely crack.

Tubes of discarded cultures are cleaned as follows: The tubes are placed upright in a basket and autoclaved. While they are still hot the cotton plugs are withdrawn, the basket is placed in a deep pail or other vessel somewhat deeper than the tubes, and the tubes, remaining upright, are filled and covered with hot water. The pail of water is heated on a burner while the vaseline rises to the surface whence after cooling it may be recovered if desired. The tubes may then be washed by the usual method.

<div align="center">SUMMARY.</div>

There has been described the use of the vaseline tube and the tuberculin syringe for the study of gas production by bacteria.

A comparison is made of some of the results obtained by the use of the method here described, the Smith fermentation tube, and the tube of Eldredge and Rogers.

The reports of CO_2 production by certain streptococci by Ayers, Rupp, and Mudge and by *Bacterium typhosus* by Nichols have been confirmed by the author's method.

The data presented serve to illustrate the accuracy and technical possibilities of the method.

In addition to economy of glassware, medium, and labor, the vaseline tube and syringe method of micro gas analysis possesses the following advantages. (1) Gas produced above either liquid or solid media may be measured and analyzed. (2) The gas produced may be measured in terms of a definite and constant quantity of medium used. (3) The vaseline tube provides a closed system from which gases do not escape into the air. (4) Separate determinations of the CO_2 produced in and above fluid media may be made. (5) Determinations may be made from very small samples of material. (6) Numerous gas analyses of the same culture may be made at various times during the growth of the culture without contaminating or destroying it. (7) Gas production may be observed under both anaerobic and controlled aerobic conditions.

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EXPLANATION OF PLATES.

PLATE 55.

FIG. 1. Withdrawing the sample of gas from beneath the vaseline plug.

PLATE 56.

FIG. 2. Sodium hydroxide solution being drawn into the syringe for the absorption of carbon dioxide.

FIG. 3. The aeration of a sample of culture for the determination of the carbon dioxide in the medium.

PLATE 57.

FIG. 4. The tube of medium slanted but still covered by a layer of vaseline.

FIG. 5. The slanted tube rotated causing the lower end of the vaseline seal to flap up, thus exposing the medium for inoculation.

FIG. 1.

(Brown   Micro gas analysis of bacterial cultures)

Fig. 2

Fig. 3.

(Brown Micro gas analysis of bacterial cultures)
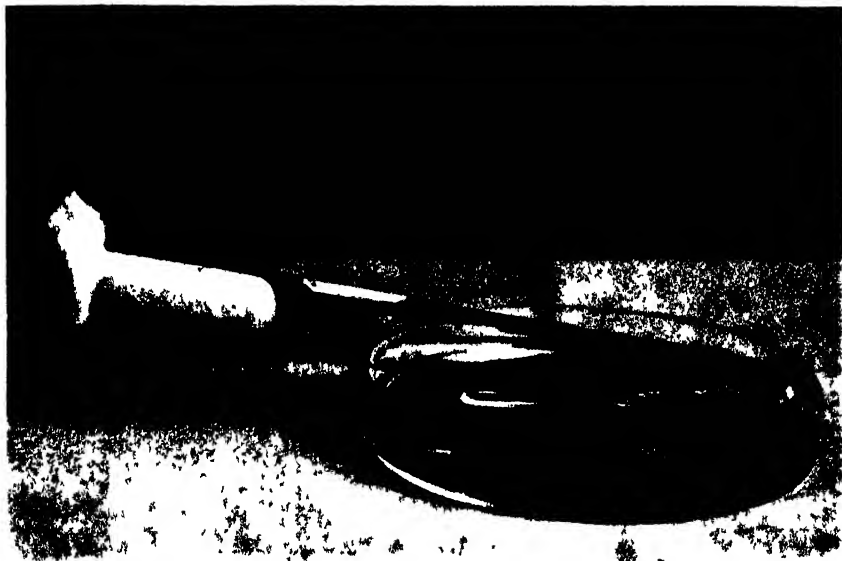
FIG 4.

FIG. 5.

(Brown: Micro gas analysis of bacterial cultures)

THE DIFFERENTIAL PRECIPITATION OF THE PROTEINS OF COLOSTRUM AND A METHOD FOR THE DETERMINATION OF THE PROTEINS IN COLOSTRUM.

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As a result of work on the appearance and disappearance of protein in the blood of young calves (1) it became desirable to determine the proteins in colostrum. The blood of a new-born calf after it has taken colostrum contains rather large quantities of euglobulin and pseudoglobulin I whereas before it has taken colostrum these proteins are absent. The evidence points to a direct absorption of the protein from the alimentary tract for two reasons, (*a*) the time interval between ingestion of colostrum and the appearance of the proteins in the blood is short, 2 to 5 hours, and (*b*) these proteins do not appear to any extent in the blood of calves which are fed milk from a cow well along in lactation. If the assumption of direct absorption is correct, and it appears to be, we should expect to find both types of globulin in colostrum.

Two groups of investigators, Crowther and Raistrick (2), Dudley and Woodman (3), and Woodman (4), working upon the relation of the proteins of milk and colostrum to each other and to similar proteins of other species or to blood have attempted the separation of euglobulin from the other globulins of colostrum. Crowther and Raistrick precipitated casein[1] with potassium alum according to the procedure of Schlossmann (5), filtered, neutralized the filtrate, precipitated the globulins with magnesium sulfate, and then dialyzed the dissolved precipitate to separate the euglobulin from the pseudoglobulin.

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[1] The term casein is used in this work to designate the protein of milk which corresponds to the English term caseinogen.

Dudley and Woodman precipitated the casein of colostrum with acetic acid, neutralized the filtrate, and then separated the globulins in the same manner as did Crowther and Raistrick.

Various procedures have been suggested for the determination of casein albumin, and total globulins in colostrum. The literature on the subject has been reviewed by Engel (6). Sebelien (7) obtained maximal values for globulin in colostrum by subtracting the value obtained for casein by precipitation with acetic acid from that obtained by precipitating with magnesium sulphate; magnesium sulphate precipitates casein and all of the globulins. A minimal value was obtained by saturation with sodium chloride which precipitates casein and part of the globulins. From our work the minimal values obtained by Sebelien probably represent the euglobulin and not any of the pseudoglobulins. Albumin was determined in the filtrate from the saturated magnesium sulfate precipitation. Tiemann (8) utilized the clay plate method of Lehmann (9) to separate the dissolved proteins of colostrum from the undissolved proteins. Results were obtained for casein which agreed very well with those obtained by the methods of Schlossmann and of Sebelien. Simon (10) has reexamined the procedures of previous investigators for the analysis of milk and obtained similar results for casein with acetic acid and alum and higher results with saturated magnesium sulphate and sodium chloride, due, as he suggests, to the precipitation of globulin by these salts. For total protein Almen's tannic acid reagent, phosphotungstic acid, and trichloroacetic acid gave essentially the same results.

The Basis for the Determination of the Proteins of Colostrum.—The general technique for the determination of the proteins of colostrum involves the direct determination of casein and the estimation of the remaining proteins by means of definite concentrations of a salt or by the use of different salts. In this work anhydrous sodium sulfate was used. Storch (11) has used sodium sulfate at room temperature in the separation of the proteins of milk. Casein was precipitated by saturated sodium sulfate, which at room temperature would be equivalent to between 14 and 19 per cent (16–20°C.). This corresponds very closely to the precipitation limits of casein found by us. Three procedures were open: Procedure A, to precipitate the casein with an acid or alum and then to add various amounts of sodium sulfate to

the neutralized filtrate, which is essentially the method of Crowther and Raistrick and Dudley and Woodman in preparing the proteins of colostrum and milk, Procedure B to precipitate casein and euglobulin together with saturated sodium chloride and from a series of precipitations with sodium sulfate on other portions of colostrum to calculate the amount of casein and euglobulin present, or Procedure C to make a series of precipitations with sodium sulfate and to precipitate the casein from the filtrates of such precipitations. It was hoped that all three procedures could be utilized in establishing one of them as the most convenient and reliable but this hope was not entirely realized. On the other hand each procedure helped in the analysis of the others. Experiments with colostrum in which Procedure A was used were variously successful and unsuccessful. The chief difficulties were the determination of the completion of precipitation of casein and the altered conditions as the result of acidification and neutralization. Procedure B was also more or less unsuccessful but the results served as a fair check on the determinations of casein and euglobulin. Procedure C has yielded reasonably consistent results.

EXPERIMENTAL.

The experimental work on the determination of the proteins of colostrum involved two phases, (a) the fractionation of colostrum with sodium sulfate and (b) the determination of casein. As the result of previous experience with blood (12) the analytical procedures were confined almost entirely to the analysis of small quantities of colostrum, 0.5 cc. diluted with 15 cc. of solution or an equivalent dilution. The measurement of such small quantities of the most concentrated samples of colostrum presented certain difficulties on account of the viscosity of the material; we were able, however, to obtain results which agreed very closely with those found with larger quantities. All samples of colostrum were centrifuged to remove most of the fat, the colostric bodies, etc.; our remarks, therefore, apply only to the "fat-free" colostrum. When sodium sulfate was used it was added to the measured sample of colostrum in concentrations which would give the desired percentage in the volume of colostrum used plus the volume of added solution. Most of the precipitations were made by the addition of known concentrations of sodium sulfate to measured quantities of

colostrum, such results were often checked by adding the salt to the diluted colostrum. The degree of dilution adopted was that found to be sufficient for blood. That the dilution was adequate was verified by experiments in which 0.5 cc. of colostrum of high protein content was added to 30 cc. of salt solution; *i.e.*, double the dilution employed in this work. All precipitations were made at 37°C. For the precipitation of casein a 10 per cent solution of acetic acid was used or a saturated solution of potassium alum. The quantity of protein precipitated was estimated by analyzing an aliquot portion of the filtrate by the Kjeldahl method. Because of the amount of material to be oxidized the usual 500 cc., or 250 cc., Kjeldahl flask was used in place of the Pyrex test-tubes employed in the work on blood, 10 cc. of sulfuric acid were used in the oxidation. With suitable precautions it is possible, however, to use the large test tubes and the micro distillation procedure introduced by Folin.

Effect of Increasing Concentrations of Sodium Sulfate upon Colostrum or Milk.—Colostrum was treated with various concentrations of sodium sulfate to determine the presence of critical zones in the curve of protein precipitated. In Tables I and II are data from experiments on colostrum and also on milk.

Sodium sulfate was added to diluted colostrum, or solutions of a given concentration to measured quantities of colostrum, so that a series of solutions which differed from each other by a small but definite amount of the salt was obtained. When precipitation occurred the filtrates were analyzed for nitrogen by the Kjeldahl method and the quantity of protein was estimated from the total nitrogen content of the colostrum. Examples of such series are contained in Tables I and II. In Table II analyses of a number of different samples of colostrum are given. These data are presented to show the nature of the deviations from the conclusions which may be drawn and also to bring out the constancy with which other phenomena occur.

Colostrum, like blood, is a mixture of proteins and other substances. Any procedure which attempts to separate the proteins of such a mixture without purification must, indeed, carry with it certain errors such as may result from adsorption, possible overlapping of precipitation limits, etc. To obtain a relative degree of constancy in any set of procedures is, we believe, to approach the minimum of error under the

conditions imposed. At present our methods for the separation of proteins are confined to precipitation with salts or acids, and occasionally alcohol.

Protein, Euglobulin, Precipitated at 14.0 to 14.2 Per Cent of Sodium Sulfate.—From a consideration of the data presented in the tables it will be seen that there is, in general, a marked change in the quantity of protein precipitated between 14.2 and 14.5 per cent of sodium sulfate

TABLE I.

Nitrogen Precipitated from Colostrum and Milk by Acidification, Saturated Sodium Chloride, and Various Concentrations of Sodium Sulfate.

	Colostrum.				Milk.	
	N in filtrate.	N precipitated.	N filtrate after acidification.	N precipitated by acid.	N in filtrate.	N precipitated.
Total N	3.226				0.551	
Alum	2.363	0.863			0.144	0.407
Acetic acid	2.408	0.818			0.124	0.427
Saturated NaCl	0.808	2.418				
Na₂SO₄						
13.5 per cent	1.992	1.234	1.159	0.883	0.553	0.007
14.0 " "	1.926	1.300	1.056	0.870	0.540	0.011
14.2 " "	1.860	1.366	1.002	0.858		
14.5 " "	1.383	1.843	0.726	0.657		
16.4 " "	0.682	2.542	0.429	0.283	0.322	0.229
17.4 " "	0.508	2.718	0.379	0.129	0.153	0.398
18.4 " "	0.445	2.781	0.338	0.107	0.136	0.415
19.3 " "	0.437	2.789	0.338	0.099	0.132	0.419
20.3 " "	0.429	2.977	0.330	0.099		
21.3 " "	0.392	2.834	0.214	0.178	0.128	0.423
23.2 " "	0.396	2.830	0.190	0.206	0.128	0.423

* Results are expressed as grams of nitrogen in 100 cc. of the original sample.

and that the quantity of protein precipitated at 14.0 to 14.2 per cent of sodium sulfate is approximately the same. The point in the curve of precipitation at which there is little change in the quantity of protein precipitated will be designated the "critical zone." A sharp critical zone is not evident when the concentrations of sodium sulfate differ by 1 per cent of sodium sulfate. If the results obtained with a difference of 1 per cent be plotted the points representing protein precipitated approximate a straight line between 12.5 and 16 per cent of sodium

sulfate with a very sharp break between 16 and 17 per cent. If, on the other hand, the results from a series of precipitations in which the sodium sulfate differs by 0.2 per cent be plotted there is almost invariably a zone between 13.5 and 14.5 per cent of sodium sulfate in which an increment of 0.2 per cent does not cause a marked increase in the quantity of protein precipitated. The critical zone usually occurs at 14.0 to 14.2 per cent of sodium sulfate. There are one or two exceptions in Table II. In the case of Sample F the critical zone occurs at 13.5 to 14.0 per cent of sodium sulfate and in the case of Sample E the critical zone is at 14.2 to 14.5 per cent.

Data obtained after the removal of casein with acid and then the addition of sodium sulfate to the neutralized filtrate did not always agree with that obtained by direct precipitation with regard to the absolute amount of protein precipitated. Whether the absolute results agreed or not the evidence of a critical zone remained and with less variation than with direct precipitation. The failure always to obtain agreement in the quantity of protein precipitated is to be laid, we believe, to the effects of acidification and neutralization. No matter what procedure is adopted the results obtained at 18.4 per cent of sodium sulfate essentially agree. In one attempt to solve the difficulties in this procedure a series of experiments was conducted in which various amounts of acetic acid were added to attain certain degrees of acidity or flocculation. After filtration the neutralized filtrates were precipitated with sodium sulfate. It was found that, in spite of the fact that various quantities of protein were precipitated by acetic acid and subsequently by 14.0 per cent of sodium sulfate, the sums of the quantities of protein precipitated by acetic acid and 14.0 per cent of sodium sulfate were practically identical and equalled the protein precipitated by sodium chloride. Furthermore, the quantity of protein precipitated by 18 per cent of sodium sulfate was essentially the same. The difficulties of this procedure with the possibilities for error do not seem to warrant its use for the determination of the proteins of colostrum.

A critical zone at 14.0 to 14.2 per cent of sodium sulfate agrees with the results obtained with blood.[2] In our discussion protein precipi-

[2] In the case of blood the limits were given as 13.5 to 14.5 per cent of sodium sulfate. From the work here presented and subsequent work on blood we would suggest 14.2 per cent of sodium sulfate as best single concentration to use: such a concentration represents an approximately molar solution of sodium sulfate.

tated up to and including the critical zone will be designated euglobulin. From the work on the determination of casein, presented below, it seems that very little, if any, casein is precipitated until 14.5 per cent of sodium sulfate has been added. Contamination of the protein precipitated at the critical zone would appear to consist essentially of pseudoglobulin I. Preparations of the protein precipitated at 14.0 to 14.2 per cent retain their precipitation reactions at these percentages, they are precipitated by dialysis and are soluble in salt solution. It may be added that under the conditions of dialysis employed a rather large percentage of salt-insoluble protein was obtained which was difficultly soluble in dilute acid or alkali in the cold.

In the case of colostrum it was not possible to verify the assumption that the protein precipitated at 14.0 to 14.2 per cent of sodium sulfate was euglobulin, as was done in the case of blood by the use of other precipitants such as carbon dioxide and saturated sodium chloride, since both euglobulin and casein are, in part at least, precipitated by carbon dioxide or saturated sodium chloride. Data obtained by the use of saturated sodium chloride agree approximately with the sum of the euglobulin nitrogen and the casein nitrogen. In Table II are given results for euglobulin obtained by subtracting the casein nitrogen from the nitrogen precipitated by saturated sodium chloride. Carbon dioxide as a precipitant of euglobulin plus casein gave rather variable results. From diluted milk, casein is completely precipitated. With colostrum, carbon dioxide apparently precipitated casein and sometimes euglobulin, at least the analytical results agree with those obtained either by acetic acid precipitation or by sodium chloride precipitation. It was thought that possibly by removal of the calcium with an oxalate or citrate consistent results could be obtained, but without success.[3]

[3] In the course of experiments dealing with the precipitation of proteins with carbon dioxide it was noted that the addition of potassium oxalate caused the opacity of the solution to disappear, due to the removal of the calcium. A similar phenomenon was observed with sodium citrate (Brown, J. H., and Howe, P. E., *Proc. Bact. Soc.*, 1921); also with sodium sulfate and magnesium sulfate. The addition of calcium chloride causes the opacity to return. This change in appearance has not been commented upon so far as we can find, it must certainly have been observed when citrating milk for infants.

The presence of Critical Zones for Colostrum of 17.4 to 18.4 and at 21 to 22 Per Cent of Sodium Sulfate.—A second critical zone is evident for both colostrum and milk at approximately 17.4 to 18.4 per cent of sodium sulfate. At these concentrations, reasoning by analogy with blood and from data obtained with milk and purified solutions of casein, pseudoglobulin I and casein are completely precipitated. Preparations of casein are precipitated completely at 18.0 per cent of sodium sulfate and dialyzed preparations of pseudoglobulin I are precipitated at 17.4 per cent of sodium sulfate.

Data obtained by fractioning the neutralized filtrate from colostrum after the precipitation of casein with acetic acid give a critical zone at 16.4 to 17.4 per cent of sodium sulfate, corresponding with the zone obtained in blood. Casein, however, appears to require a slightly higher concentration of sodium sulfate, 18.0 per cent, to insure complete precipitation under all conditions. To accept 18.0 or 18.4 per cent of sodium sulfate as the concentration which represents the completion of precipitation of casein and pseudoglobulin I tends to introduce an added element of error in the pseudoglobulin II determination. Experiments in which the casein has been removed before precipitation with sodium sulfate indicate that the error for pseudoglobulin II introduced at 18.0 or 18.4 per cent is relatively small.

A third critical zone at 21 to 22 per cent of sodium sulfate is present in colostrum and milk. The difference between the results obtained at 18.4 and 21.5 per cent of sodium sulfate is usually small, it is practically negligible in the case of milk. The addition of saturated sodium sulfate at 37°C. to colostrum precipitates all of the proteins; 30 parts of saturated sodium sulfate to 1 part of colostrum are sufficient.

From the results presented and observations made in the course of the work which can be considered as contributary evidence it appears that colostrum may be subdivided by fractionation with sodium sulfate into four protein groups, euglobulin, pseudoglobulin I and casein, pseudoglobulin II, and albumin. Non-protein nitrogen is present in the filtrate from precipitation with saturated sodium sulfate or trichloroacetic acid.

Determination of Casein in Colostrum.—In the determination of the proteins of colostrum the estimation of casein presents certain difficulties and at the same time complicates the estimation of the other

proteins. Casein is precipitated by concentrations of salts which also precipitate one or both of the pseudoglobulins; saturated magnesium sulfate, ammonium sulfate, precipitation limits 2.2 to 3.6 saturation (13) and sodium sulfate, precipitation limits 16 to 18.4 per cent. On the other hand precipitation with acids, the characteristic property of casein, is a property of euglobulin and saturation with sodium chloride is a property of these two proteins.

In the work of Crowther and Raistrick and of Dudley and Woodman the procedures adopted for the separation of casein, direct precipitation with acetic acid or alum, may or may not succeed according to the quantity of acid or alum added. In case sufficient acid or alum is added to redissolve the euglobulin which is precipitated at low concentrations of these substances separation is essentially complete. The addition of either precipitant to colostrum to the point of ready flocculation does not, however, insure the complete precipitation of casein or the complete solution of euglobulin. A clear supernatant fluid is generally the best evidence of complete precipitation of casein but this has not always been so in our work. The isoelectric point of euglobulin is pH 5.52 (14) and for casein pH 4.4 (15). The point of maximal insolubility has been shown to be at or close to the isoelectric point. From original mixtures of proteins precipitation may occur at a hydrion concentration slightly removed from the isoelectric point. Hardy (16) has shown that euglobulin is dissolved at a point at which methyl orange is slightly pink, which represents a hydrion concentration of between pH 4 to 5. We can assume, therefore, that recently precipitated euglobulin will be practically dissolved by acid at the isoelectric point of casein. When acidifying with acid to precipitate casein it is desirable to carry the reaction approximately to the isoelectric point of casein. A ready means of doing this is not available since, in addition to the opacity of the colostrum, the indicators which can be used do not show sharp color changes at the desired range of hydrion concentrations. Reasonably satisfactory results have, however, been obtained with methyl orange.

The practical difficulty in the separation of casein from the other proteins of colostrum is, then, the means of knowing when sufficient acid or alum has been added to precipitate and redissolve the euglob-

TABLE I.

Data Showing the Effect of Acidification and the Addition of Sodium Chloride and Various Concentrations of Sodium Sulfate when Added to Colostrum.*

	Sample A			Sample B			Sample C			Sample D		
	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.
Total N	1.199			2.016			2.365			2.504		
Alum	0.578†			0.677†			1.053†			0.875†		
Acetic acid	0.573†			0.668†			1.072†			0.866†		
NaCl, saturated	0.876			1.525			1.717			1.664		
Euglobulin, calculated from NaCl precipitation	0.300			0.847			0.651			0.811		
Na₂SO₄												
12.5 per cent	0.215	0.358	0.619†	0.592	0.751	0.673†	0.356	0.925	1.084†	0.559	1.084	0.861†
13.5 " "	0.254	0.354	0.584†	0.692	0.772	0.657†	0.394	0.909	1.062†	0.853	0.793	0.858†
14.0 " "	0.258	0.358	0.576†	0.745	0.593	0.678†	0.416	0.883	1.066†	0.892	0.750	0.853†
14.2 " "	0.284	0.345	0.563†	0.865	0.520	0.630†	0.536	0.751	1.078†	1.007	0.644	0.853†
14.5 " "	0.778	0.172	0.242	1.581			1.491	0.384	0.490	1.873		
17.4 " "	0.893	0.175	0.124	1.700			1.911	0.273	0.171	2.052		
18.4 " "	0.991	0.154	0.047	1.773			2.083	0.273	0.009	2.188		
21.5 " "												
Albumin N + non-protein N	0.201			0.243			0.282			0.316		
Non-protein N				0.081			0.154			0.073		
Euglobulin	0.258			0.745			0.416			0.892		
Casein	0.576			0.678			1.066			0.853		
Pseudoglobulin I	0.159			0.277			0.429			0.307		
" II				0.073			0.172			0.136		
Albumin	0.098			0.162			0.128			0.243		

TABLE II.

Chloride and Various Concentrations of Sodium Sulfate when Added to Colostrum.*

	Sample E					Sample F			Sample G			Sample H		
	N precipitated.	Filtrate N after addition.	N precipitated by acetic acid.	Filtrate N after precipitation with alum.	N precipitated by alum.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.
Total N	2.839					3.116			3.119			3.213		
Alum	1.343†					0.832†			1.110†			0.911†		
Acetic acid	0.861†					0.843†			1.110†			1.015†		
NaCl, saturated	2.086								2.031			2.340		
Euglobulin, calculated from NaCl precipitation	1.305								1.009			1.688		
Na₂SO₄														
12.5 per cent	0.746	1.224	0.849†	1.224	0.849†	0.728	1.092	1.282	0.662	1.403	1.054†	1.363	1.173	0.677†
13.5 " "	0.925	1.113	0.781†	1.105	0.789†	1.175	1.092	0.849†	1.020	1.070	1.029†	1.684	0.934	0.636†
14.0 " "	1.129	0.964	0.726†	0.939	0.751†	1.619	0.708	0.789†	1.200	0.897	1.022†	1.776	0.785	0.652†
14.2 " "	1.160	0.862	0.797†	0.866	0.793†	1.999	0.529	0.588	1.285	0.853	0.981	2.049	0.691	0.473
14.5 " "	2.239	0.316	0.274	0.418	0.162	2.707	0.303	0.106	2.407	0.490	0.213	2.684	0.435	0.094
17.4 " "	2.422	0.303	0.094	0.307	0.090	2.711	0.273	0.132	2.671	0.119	0.329	2.736	0.384	0.093
18.4 " "	2.474	0.277	0.068	0.273	0.072	2.813	0.196	0.107				2.802	0.260	0.141
21.5 " "														
Albumin N + non-protein N	0.345					0.303			0.448			0.401		
Non-protein N						0.124			0.171			0.171		
Euglobulin	1.129					1.184			1.200			1.776		
Casein	0.781					0.840			1.022			0.652		
Pseudoglobulin I	0.505					0.677						0.308		
" II	0.032					0.102						0.066		
Albumin						0.179			0.277			0.230		

* Results are expressed as grams of nitrogen in 100 cc. of centrifuged colostrum.

Columns headed "Filtrate N after acidification" refer to nitrogen in the filtrate from the original colostrum. "N precipitated by acetic acid (or alum)" refer to the nitrogen precipitated from the filtrate from the original sodium sulfate precipitation by the addition of acetic acid (or alum).

† Values to be compared with regard to precipitation of casein with acid or alum directly from diluted colostrum and after removal, or partial removal, of euglobulin with sodium sulfate.

ulin[4] without creating an excess of precipitant which will redissolve the casein. This difficulty is increased in the case of colostrum because of the variable concentration of protein which prevents the possible predetermination of the amount of acid or alum which will produce the desired result such as has been determined for milk. Another factor is introduced when using small amounts of colostrum for analysis in that it is desirable to add the acid or alum in a rather concentrated form in order that the volume of the solution will not be increased appreciably.

As the result of experimentation with the direct precipitation of casein with acetic acid and alum the acceptance of data upon colostrum obtained in this way without other confirmation does not seem justified. The results presented show, however, that the direct precipitation of casein may be, and usually is, complete. Samples E and H, Table II, are examples of cases in which it was impossible to obtain consistent results by direct precipitation.

The method finally adopted for the determination of casein is to precipitate it from solution by acidification with acid or alum after the euglobulin has been removed with sodium sulfate. The results of representative experiments are contained in Table II. The point to be emphasized with regard to casein from the data presented is that the quantity of protein precipitated with acetic acid, representing casein (similar results have been obtained with alum, Sample E), when added directly to diluted colostrum or to the filtrate from colostrum precipitated at approximately 14.2 per cent of sodium sulfate is essentially the same. These data are indicated by a dagger (†) in the table. The amount of acetic acid which must be added is less than that required for the original colostrum, as a routine procedure 3 drops of 10 per cent acetic acid have been found to be sufficient.

Procedure for the Analysis of Colostrum.—The procedures adopted for the analysis of colostrum or milk are based on the considerations discussed above. Material precipitated by 14.2 per cent of sodium sulfate, *i.e* 14.2 gm. of sodium sulfate contained in 100 cc. of solution, is considered to be euglobulin; that precipitated at 18.4 per cent of sodium sulfate is held to represent euglobulin, pseudoglobulin I, and

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[4] It is possible to add a sufficient excess of either acid or alum such that there will not be a visible precipitation of euglobulin before solution takes place.

casein; that at 21.5 per cent of sodium sulfate as precipitating pseudo-globulin II in addition to the proteins given for 18.4 per cent; the remainder of the nitrogen is considered to be albumin and non-protein nitrogen. Casein is determined by acidification of the filtrate from the 14.2 per cent sodium sulfate precipitation. Non-protein nitrogen is determined by precipitation with trichloracetic acid.

For duplicate precipitations we suggest precipitation of euglobulin at 14.0 and 14.2 per cent of sodium sulfate. If, as sometimes happened, the values so obtained do not agree within reasonable limits, the value at 13.5 usually agrees with that at 14.0 per cent. The duplicate determinations also give two values for casein. As a check on the casein determination a direct precipitation can be made. Precipitations at 17.4 and 18.4 per cent usually agree but not always so, in general the results at 18.4 per cent have agreed with all other procedures. Instead of 21.5 per cent of sodium sulfate a pair of precipitations can be made with 21 and 22 per cent of sodium sulfate. For very viscous samples of colostrum the sample can be weighed out and diluted with 1 per cent sodium sulfate and then weighed amounts of sodium sulfate added to aliquot portions; this procedure requires correction to volume, if the results are to be expressed on the volume basis.

The details of the procedure are as follows: 1.0 cc. portions of centrifuged colostrum are measured into large test-tubes and 30 cc. of the required concentration of sodium sulfate added. The following concentrations of sodium sulfate are prepared, 14, 14.5, 14.65, and 15 per cent sodium sulfate for 13.5, 14.0, 14.2, and 14.5 per cent of sodium sulfate, final concentrations, respectively, when added to colostrum for use at the euglobulin zone; 18 and 19 per cent for 17.4 and 18.4 per cent of sodium sulfate, respectively, at the end of the pseudoglobulin I precipitation; and 21.7, 22.2, and 22.7 per cent for 21, 21.5, and 22 per cent of sodium sulfate, respectively, for precipitation of the total globulins. 5 per cent trichloroacetic acid is used for the complete precipitation of proteins in the non protein nitrogen determination. For the determination of casein, 3 drops of 10 per cent acetic acid are added to the filtrate from the sodium sulfate precipitations at the euglobulin critical zone and after the precipitate has settled the solution is filtered and the nitrogen content of the filtrate

is determined. The precipitations, with the exception of the tri-
chloroacetic acid precipitation, must be carried out at 34°C., or incuba-
tor temperature. The test-tubes are stoppered and permitted to stand
until the precipitate has settled, and then filtered. We usually shake
up a precipitate after it has settled and allow it to settle once more.
Filtrations are conducted into other test-tubes on a dry filter and the
funnels are covered with watch-glasses. 5 cc. portions of the filtrates
are taken for analysis by the Kjeldahl procedure.

The following calculations can be made:

Total N, determined directly.

Euglobulin N   = Total N — N in filtrate from precipitation with 14.2
     per cent sodium sulfate.

Casein N (1)   = N in filtrate from 14.2 per cent sodium sulfate — N
     in filtrate after acidification.

Casein N (2)   = Total N — N in filtrate after acidification with acetic
     acid.

Casein N + pseudo-
    globulin I N   = N in filtrate from precipitation with 14.2 per cent of
     sodium sulfate — N in filtrate after precipitation
     with 18.4 per cent sodium sulfate.

Pseudoglobulin I N   = N found for casein + pseudoglobulin I — casein N.

Pseudoglobulin II N   = N in filtrate from precipitation with 18.4 per cent
     sodium sulfate — N in filtrate from precipitation
     with 21.5 per cent sodium sulfate.

Albumin N   = N in filtrate from precipitation with 21.5 per cent
     sodium sulfate — non-protein N.

Non-protein N   = N in filtrate from precipitation with 5 per cent
     trichloroacetic acid.

It will be noticed that there are but two direct determinations,
total nitrogen and non-protein nitrogen; the other determinations
depend upon analysis of the filtrate after precipitation and the calcu-
lation of the protein precipitated by subtraction. The errors of such
methods are appreciated but the procedures outlined seem most nearly
to meet the requirements for the differential determination of the
proteins of colostrum.

DISCUSSION.

Data have been presented which indicate that there are consecutive
concentrations of sodium sulfate which, when added to diluted colos-
trum or milk, do not cause a marked increase in the quantity of protein

precipitated and define a critical zone, and that on either side of such a zone a small variation in the quantity of sodium sulfate added results in a relatively large difference in the quantity of protein precipitated. Critical zones are at 14.0 to 14.2, 18.0 to 18.4, and 21 to 22 per cent of sodium sulfate. Similar zones have been obtained in the neutralized filtrates of colostrum from which the casein has been removed with acetic acid. Casein is not precipitated by 14.0 to 14.2 per cent of sodium sulfate but is precipitated by 18.0 to 18.4 per cent sodium sulfate; it is also precipitated by acidification of the diluted colostrum with acetic acid or alum or by the acidification of the filtrate from a precipitation with sodium sulfate. If the sodium sulfate precipitation be made at a concentration less than 14.5 per cent the casein can be recovered completely by subsequent acidification of the filtrate.

As the result of the above observations it is suggested that there exists a basis for the quantitative determination of the various proteins of colostrum or milk. The material separated at the various concentrations of sodium sulfate when added to colostrum in the proportion of 31:1 is considered to consist of the following proteins or mixtures of proteins: (a) at 14.0 to 14.2 per cent of sodium sulfate, euglobulin; (b) at 18.0 to 18.4 per cent of sodium sulfate, euglobulin, pseudoglobulin, I, and casein; and (c) at 21 to 22 per cent of sodium sulfate, euglobulin, pseudoglobulins I and II, and casein.

With the values for casein and non-protein nitrogen determined independently we are, then, able to calculate the quantities of the various proteins present in colostrum.

It is necessary to consider the justification for the assignment of names to the proteins of colostrum, with the exception of casein, which are used to designate the proteins of blood.

Euglobulin.—The evidence for considering protein precipitated up to and including 14.5 per cent of sodium sulfate in blood as consisting essentially of euglobulin has been presented (11). Similar precipitation limits of a protein in colostrum even though the reaction be slightly different may be taken as presumptive evidence of identity. The following facts tend to support this presumption: (a) precipitation reactions of the purified protein; precipitation by dialysis and by acidification; (b) the proof offered by Crowther and Raistrick, Dudley

and Woodman, and Woodman which indicates that the euglobulin of serum and of the colostrum of the cow are identical. These authors support the contention of Chick (17) and Hartley (18) that the protein portion of euglobulin and pseudoglobulin is identical. Chick suggests that euglobulin may be a mechanical complex of a lipoid and pseudoglobulin. The proof with regard to the identity of the protein portion of euglobulin and pseudoglobulin as far as the yield of amino-acids and the rate of racemization are concerned is convincing; we still lack the biological proof, anaphylactic reaction, of the identity of these two fractions such as has been furnished by Wells and Osborne (19) for the individuality of casein, albumin, globulin, and the alcohol-soluble protein of milk. In the work of Wells and Osborne no attempt was made to distinguish between pseudoglobulin and euglobulin of milk, which would be difficult considering the small quantities of globulin present. Osborne (20) did find phosphatide phosphorus present in the globulin fraction. The relatively large percentage of phosphorus present would suggest that the globulin was euglobulin or a mixture of euglobulin and pseudoglobulin. The limited experience which we have had with milk suggests that euglobulin is sometimes present and in other samples of milk it is practically absent.

From the facts in hand we believe that there is sufficient justification for the assumption that the protein precipitated at 14.0 to 14.2 per cent of sodium sulfate consists essentially of a protein, or protein complex, usually designated as euglobulin and that the euglobulin of colostrum is the same as that of blood.

Pseudoglobulin I.—The evidence for considering the protein precipitated between 14.2 and 18.4 per cent of sodium sulfate as consisting essentially of casein and pseudoglobulin I is based upon our results and that of the same group of investigators mentioned above. The distinction between two pseudoglobulins is based largely on our work. Supporting evidence of Crowther and Raistrick, Dudley and Woodman, and Woodman, relates to fractions which would include both of the pseudoglobulins. Here as with euglobulin it was found that the pseudoglobulin of colostrum had the same characteristics as that of blood. The question of the identity of pseudoglobulin and euglobulin must be considered here also.

Casein.—The presence of casein in the fraction precipitated between 14.2 and 18.4 per cent of sodium sulfate must rest upon results presented in our experimental work and that of Storch; that the material is casein is indicated by the purified product which is precipitated by acids and coagulated by rennin in the presence of added calcium salts.

Pseudoglobulin II.—The fraction between 18.4 and 21 to 22 per cent of sodium sulfate does not have any supporting evidence except that presented in connection with this work and the work on blood. That we are dealing with a distinct protein rests on a rather meager basis at present. In colostrum or in blood there is very little of this protein present. The evidence is, however, such that we feel that the fraction should be retained as a probable entity until proof can be presented showing that it is not a particular protein.

Albumin of colostrum and milk has been shown to be chemically different (2, 3, 4) and the albumin of milk to be biologically different (19) from the albumin of blood. The albumin of colostrum and milk should, therefore, be designated lacto-albumin. Data obtained with new-born calves (unpublished) indicate that there is not an increase in the albumin content of the blood at the same time as the increase in the globulins occurs. The increase in albumin which does occur later is a gradual one, suggesting the formation or accumulation of albumin by the calf.

SUMMARY.

A basis for the differential estimation of the proteins of colostrum and milk has been presented, and a procedure for the analysis of colostrum suggested.

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